TOWARDS A BIOPHYSICAL UNDERSTANDING OF HALLUCINOGEN ACTION

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By this it appears how necessary it is for any man that aspires to true knowledge, to examine the definitions of former authors; and either to correct them, where they are negligently set down, or to make them himself. For the errors of definitions multiply themselves according as the reckoning proceeds, and lead men into absurdities, which at last they see, but cannot avoid, without reckoning anew from the beginning.

– Hobbes, Leviathan, 1651.

ACKNOWLEDGMENTS

A work of this scope would take a document of near equal length to thank all the family, friends, mentors, and influences I have had in my life that have led me to this point, so I will attempt to limit myself to the most relevant: first my parents, for supporting me, both lovingly and financially, as well as letting me find my own trouble, solutions, and personal philosophy; my brother for being a good friend, not torturing me too much, and grudgingly letting me play with his toys; my thesis advisor Dr. David Nichols for letting me figure things out on my own and treating me as a peer in the end; my thesis committee for being patient with me as I discovered my own projects and also for challenging me to think critically; my undergraduate research advisor Dr. John Naleway for being patient with me and teaching a variety of laboratory techniques; and all of my friends, past, present and future, who make this meager life worth living and so very enjoyable.

PREFACE

> *[T]here are two sorts of intellectual labyrinths into which all thinking people are sooner or later drawn.[…]One is the composition of the continuum, which is to say, what is matter made of, what's the nature of space, et cetera. The other is the problem of free will: Do we have a choice in what we do? Which is like saying, do we have souls?*
>
> - Neal Stephenson from the novel The System of the World, 2004

The two intellectual labyrinths described in the above quotation remain contemporaneous with the modern study of consciousness. As a scientist, I feel compelled to explore my slice of the continuum for a mechanistic understanding of how mental processes work at the molecular level, studying the biophysical and biochemical systems from the bottom-up. Others approach the questions of the phenomenology and/or etiology of consciousness from a top-down approach, *viz.* behavioral and systems neuroscience. Unfortunately, many aspects of this scientific ontology do not blend to a continuum but remain discrete areas of understanding. Unifying these disciplines remains a great task.

This work is a companion to that of my colleague Jason Parrish, so I similarly feel compelled to address in this preface the issue of free will as it relates to neuropharmacology. The question of whether the mind and body are separate is no longer relevant. That our biology can affect our psychology and *vice versa* is well established. Descartes may have skirted the greater issue in an attempt to avoid religious persecution by not asking whether the mind is greater than the sum of its parts. The alchemists, mystics, and theologians would have us believe that our viscera are suffused with a quintessence; a fifth element, spirit or soul, the seat of our uniqueness of being. If the mind is indeed a biophysical and biochemical engine that functions based solely on

deterministic principles, then there exists the possibility that existence is purely deterministic and that free will does not exist.

Yet as a scientist, the question of whether or not free will exists is moot, as it is likely that proof of its existence is beyond the scope of scientific empiricism. Instead, we must recognize that the *illusion* of free will is what is important. As with many scientific questions, it is a matter of perspective, or where we define our boundary conditions. That we are able to make choices and can willfully direct behavior is apparent. Regardless of whether or not free will exists we are responsible for our actions. Yet we should not fret upon every choice presented to us, for the future is always unknown. Much of our emotional processing occurs at a non-conscious or sub-conscious level, yet we can consciously learn to adapt, control, ignore, or embrace our feelings. It is our awareness that allows us to observe that a choice may exist and determine when to accept the choice that has already been made or attempt to act willfully.

The altering of our awareness is what makes hallucinogens such unique tools for the study of consciousness. Their effect is not limited simply to changes in our sensory perceptions, *e.g.* sight or hearing, but more importantly our awareness of self, *viz.* boundaries between self and other. These temporary changes to our consciousness induced by hallucinogens can have long lasting effects on our awareness, particularly in the recognition that our senses are fallible, including our sense of self. The recognition that the boundaries of the self are plastic can be both powerful and frightening, leading towards epiphany or existential crisis. Indeed, the ability to reconstruct a sense of self dynamically is understood by some to be integral to mental health. Hallucinogens further allow us to probe the brain mechanisms that contribute to these aspects of consciousness in the brain. It is my hope that the study of hallucinogens will ultimately help to bridge the fields of neuroscience and the study of consciousness into a continuous spectrum of understanding.

> *If the doors of perception were cleansed everything would appear to man as it is, infinite. For man has closed himself up, till he sees all things thro' narrow chinks of his cavern.*
>
> – William Blake The Marriage of Heaven and Hell, 1793

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
Å	angstrom (10^{-10} meter)
ΔpEC50	change in negative log EC50 values
ΔG°	standard Gibbs free-energy (of binding)
ΔΔG°	change in standard Gibbs free-energy (of binding)
ΔpK_i	change in negative log of K_i equilibrium constant
π-π	induced dipole interaction between two π-electron shells/distributions
3-D	3-dimensional
ANOVA	analysis of variance
bRho	bovine rhodopsin
BGH	bovine growth hormone
CNS	central nervous system
d-LSD	*dextro*-lysergic acid diethylamide
DMEM	Dulbecco's modified Eagle's media
DNA	deoxyribonucleic acid
DOB	4-bromo-2,5-dimethoxyphenylisopropylamine
DOI	4-iodo-2,5-dimethoxyphenylisopropylamine
DOM	4-methyl-2,5-dimethoxyphenylisopropylamine
DMT	*N,N*-dimethyltryptamine
DET	*N,N*-diethyltryptamine
DPT	*N,N*-diisopropyltryptamine
EC50	effective concentration for 50% of maximal response (potency)
EF-1α	elongation factor 1α
EL	extracellular loop

GPCR	G-protein coupled receptor
Hh2A	HEK-293 cells with stable heterologous expression of h5-HT_{2A} receptors
IL	intracellular loop
Int.Act.	intrinsic activity (efficacy)
K_i	inferred equilibrium binding affinity value
MD	molecular dynamics
NMR	nuclear magnetic resonance (spectroscopy)
NTV	ensemble of number of atoms, temperature and volume kept constant
PCR	polymerase chain reaction
PEA	phenethylamine/phenylethylamine
PI	phosphatidylinositol/phosphatidylinositide
PIA	phenylisopropylamine
RMS(D)	root mean square (deviation)
SAR	structure-activity relationship
SEM	standard error of the mean
TLC	thin layer chromatography
TM	transmembrane

One letter codes and abbreviations for amino acids used in this work:

A or Ala	alanine
C or Cys	cysteine
D or Asp	aspartic acid
E or Glu	glutamic acid
F or Phe	phenylalanine
G or Gly	glycine
N or Asn	asparagine
P or Pro	proline
S or Ser	serine
T or Thr	threonine
W or Trp	tryptophan

ABSTRACT

Braden, Michael Robert. Ph.D., Purdue University, May, 2007. Towards a Biophysical Understanding of Hallucinogen Action. Major Professor: David E. Nichols.

The serotonin 2A (5-HT_{2A}) receptor is necessary for the psychopharmacological actions of the serotonergic hallucinogens such as LSD. An exploration of the biophysical actions of hallucinogens at the 5-HT_{2A} receptor may be useful in understanding their unique psychological effects, particularly in the elucidation of structure-activity relationships for developing potent receptor- and functionally-selective 5-HT_{2A} agonists. Experiments were undertaken to optimize, validate, and explore the utility of an *in silico*-activated human 5-HT_{2A} receptor homology model developed previously in our laboratory. In the original model, a number of receptor-ligand interactions were observed. The lack of strong empirical support for several of the interactions indicated in the original modeling provided opportunities to explore further the topology of the 5-HT_{2A} receptor binding site, which also provides support for the model itself. The first section of this work describes a qualitative use of our h5-HT_{2A} receptor homology model to provide a molecular basis for the pharmacological characterization of psychoactive phenylalkylamine hallucinogens. Subsequent sections detail a systematic iterative approach to explore several of the receptor-binding interactions observed in virtual docking simulations to our h5-HT_{2A} receptor model. Data were generated by site-directed mutagenesis of h5-HT_{2A} receptor residues, with binding and functional assays. Mutation of Phe6.51(339) and Phe6.52(340) to leucine residues gave results consistent with previous studies that indicated an aromatic interaction between Phe6.52(340) and 5-HT_{2A} receptor agonists. Importantly, a novel role for Phe6.51(339) was identified, where it was found to interact with a new class of 5-HT_{2A} receptor agonists. Data from the mutation of Gly5.42(238), Ser5.43(239), and Ser5.46(242) to alanine residues are

consistent with the orientations of phenylalkylamines, tryptamines, and ergolines observed in the original development of our h5-HT_{2A} receptor model. Mutation of Ser3.36(159) and Thr3.37(160) to alanine residues did not, however, provide data to support the hypothesis of hydrogen bond interactions between these residues and the 2-methoxy of phenylalkylamines. Similarly, data from the mutation of Asn6.55(343) failed to support the hypothesis of its interaction with the carbonyl of ergolines. Overall, the data from this work provide strong evidence to support the topology of our h5-HT_{2A} receptor homology model, although further refinement of the model remains necessary.

CHAPTER 1. INTRODUCTION

1.1. Use of hallucinogens

Hallucinogens are remarkable compounds. An amount of LSD no larger than a grain of salt can strongly alter the consciousness of an individual for 8 to 12 hours. A slightly larger amount of the psychedelic amphetamine DOB can last up to 36 hours. Slight changes in the structure of these compounds can dramatically alter the subjective effects they elicit. Without even ingesting these compounds, they can simultaneously conjure wonder, reverence, and respect and yet also anxiety, fear, and/or ire. Some people consider these substances sacraments, methods for generating spiritual experiences and connecting to the divine. Others view them as recreational drugs, a rollercoaster ride of sensory distortions and cognitive tomfoolery. Still others view these as cheap tricks, shortcuts to enlightenment, drugs of abuse, and believe them to be too dangerous for the public good.

Yet another view exists for those who recognize the extraordinary actions of these compounds on a complex phenomena, namely, throwing a wrench into the mechanisms of consciousness. In this perspective, these compounds are unique tools for studying the underlying processes that make up what we define as self and the complex character of how that self experiences and interacts with the world around it. Thus, much as the practice of medicine develops from the analysis of disease states or disorders, a mechanistic understanding of the actions of these compounds may help in understanding the etiology of fundamental aspects of consciousness itself. This is the general goal to which this work seeks to contribute.

Numerous books, theses, manuscripts and scientific review articles have covered the topics of hallucinogens, of which this introduction is a mere shadow (Cohen, 1967; Nichols, 1981; Nichols, 1986; Ott, 1993; Strassman, 1995; Nichols, 1997; Marek and

Aghajanian, 1998; Aghajanian and Marek, 1999; Nichols, 2004). Many terms have been coined or used to describe the compounds studied in this work. Due to the complex nature of these molecules, most terms are simplified descriptions of the effects the drugs are expected to elicit, even though their meaning has often changed or no longer applies. However, these terms are also useful reference points for a historical perspective on the use of these substances.

"Psychotomimetic" was a term popular for many "drugs of abuse" in the early study of the effects of these compounds, as the drug-induced states were thought to resemble common psychoses, namely schizophrenia (Osmond and Smythies, 1952; Osmond, 1957; Hoffer, 1967). Similarly, the terms "deleriant" or "dissociative" have sometimes been used, for the drug-induced states, especially at higher doses, can cause various degrees of disconnection from "reality." The effects of these compounds were considered useful in understanding the psychoses they mimicked and to aid in developing treatments. This marked a shift in the zeitgeist towards a biochemical basis of psychological disorders that continues today. As schizophrenia research has developed, it has appeared that a direct association between the effects of these compounds and this disorder may be tenuous, although connections still remain. For instance, part of the action of some newer atypical antipsychotics comes from their blockage of the same receptors at which the hallucinogens act (Seeman, 2002; Horacek *et al.*, 2006).

These same compounds thought to induce psychosis show clinical utility in the treatment of some psychological disorders, and may be used to promote a conscious reintegration of the self after the so-called drug-induced psychotic state. Early research investigated the use of LSD and DPT as adjuncts in the treatment of alcoholism, with varied success (Hoffer, 1967; Kurland *et al.*, 1967; Grof *et al.*, 1973b). A comprehensive review of these studies suggests that, due to a variety of divergent factors in the previous studies, the utility of LSD in the treatment of alcoholism is inconclusive, although it does not discount the need for further research in this area (Mangini, 1998). A single LSD experience by terminal cancer patients was also found useful in improving the mood/outlook, reducing fear and anxiety, increasing communication with family members, and reducing the need for pain-relieving medication (Kast and Collins, 1964;

Kast, 1966; Pahnke *et al.*, 1969; Kast, 1970; Grof *et al.*, 1973a; Kurland, 1985). How well the patients responded was also correlated with the intensity of the LSD experience, with the most intense experiences producing the most dramatic post-therapy benefits (Pahnke *et al.*, 1970). Recently, psilocybin, the major component of "magic mushrooms", has been investigated in the treatment of obsessive compulsive disorder (Moreno and Delgado, 1997; Delgado and Moreno, 1998a; Delgado and Moreno, 1998b) and in the easing the anxiety of end-stage cancer patients (see http://www.heffter.org). Both LSD and psilocybin may be effective in the treatment of cluster headaches (Sewell *et al.*, 2006).

Psilocybin has also been used to explore another aspect of the subjective experience of these compounds. The "Good Friday experiment" and a recent extension of this classic experiment show that psilocybin can induce dramatic spiritual experiences in people with a demonstrated commitment to spiritual practices, with long lasting benefits to the individual and indistinguishable from spontaneous mystical experiences (Pahnke, 1963; Doblin, 1991; Griffiths *et al.*, 2006). The historical use of these substances, far predating modern scientific and recreational usage, was primarily in shamanic or spiritual rites and ceremonies (Ott, 1993). Meso-Americans would drink *yagé* or *ayahuasca*, concoctions of the "Jaguar vine" *Banisteriopsis caapi* with admixtures of other plants that contained the hallucinogen DMT or other potent plant alkaloids. The *B. caapi* vines provided a source of beta-carbolines, compounds that inhibit monoamine oxidase, allowing the potent hallucinogen DMT to be orally active. Similarly, other native Americans used psychoactive mushrooms or cacti in their ceremonies to commune with their gods or ancestors, namely *teonanácatl* ("flesh of the gods"; *Psilocybe sp.*), *péyotl* (peyote or "furry thing"; *Lophophora williamsii*), and *San Pedro* (*Trichocereus sp.*). Up to the fourth century CE, Greeks were initiated into the rites of the Eleusinian Mystery, a celebration of Demeter, the goddess of fertility and her role in the changing of the seasons, by ingesting a drink called κψκεον (*kykeon* or "mixture"). Officially a mixture of barley, water and an otherwise inactive mint, initiates were sworn to secrecy by penalty of death not to report to non-initiates the "Greater Mystery" that was revealed. This experience was nevertheless described as significant

and life-changing. Several recent researchers believe that the mixture contained additional strongly psychoactive ingredients, namely ergot or mushrooms, although there is much disagreement as to the possibility of proving these hypotheses (Wasson *et al.*, 1978; Ott, 1993; Webster *et al.*, 2000).

Many of the traditional uses of these plant compounds and mixtures continue today with similar spiritual goals in mind, both by native cultures and non-native Westerners. In this vein, “entheogen” is a neologism coined by a number of ethnobotonists and classicists in 1979 that literally means "that which causes God to be within an individual" or "creating the divine within" (Ruck *et al.*, 1979). They felt the conventional terms “psychedelic” and “hallucinogen” (described below) were inadequate to convey the mystical aspects of use of these compounds. This term, however, connotes aspects of the subjective experience of these compounds beyond the scope of this current work and is unlikely to ever gain acceptance in scientific circles. Similarly, the rarely used term “phanerothyme” was coined by Aldous Huxley to connote the spiritual connection of these compounds in his experiences with mescaline, the active component of the peyote cactus.

In response to Huxley’s use of phanerothyme, his psychiatrist friend Humphry Osmand, who introduced Huxley to mescaline, coined the term “psychedelic.” This is another neologism that means “mind manifesting” (Osmond, 1957). For the scientific investigation of how these compounds affect and reveal aspects of consciousness, the use of this term is desirable. Unfortunately, its use has become pejorative as it carries much social baggage due to popularization of these compounds using this word in the 1960s and 1970s (Jesse, 2000). As a result, “psychedelic” is more associated with the abuse of these compounds rather than their clinical or scientific utility.

Any of the above terms are applicable to some degree. Finding a term general enough to describe the aspects of these compounds as it relates to neuropharmacological research, yet without maligned or misappropriated connotations, is quite preferable, although a nigh impossible task.

The term “hallucinogen” is commonly used today in the scientific literature, even though this descriptor is inaccurate in its typical translation, namely “seeing illusions.”

Hallucinogen was first used in the 1950s to describe these drugs, as they generated hallucinations, although usage of the word hallucinate dates back to the 1600s. The original meaning of this word essentially translates to "wandering of the mind", although it often is used to mean "seeing something that isn't there" or "having illusions" (Online Etymology Dictionary, http//etymonline.com, © 2001 Douglas Harper). The original etymology of hallucinogen, with some specifications, is utilized in this work, namely that the effects of these compounds can ideally be attributed to the "wandering" of particular core processes in the brain that construe fundamental aspects of "normal" consciousness.

Many effects are reported due to the ingestion of hallucinogens (Hollister, 1984). Body effects include lethargy, nausea, vomiting, muscle weakness, blurred vision, and a tingling, itching or burning of the skin. Effects on sensory perceptions can include shifts in colors or shapes of objects, difficulty focusing on an object, difficulty in the definition of object boundaries, enhanced or distorted hearing, and, rarely, a blending of the senses (synesthesia; *e.g.* "seeing" a sound). Mental effects can include difficulty communicating and expressing thoughts, a volatile shift in moods, anxiety, an altered perception of time, a dream-like state, depersonalization, spiritual experiences, dissociation, and visual illusions.

A subtle but important distinction must be made to distinguish the more general effects of hallucinogens and a mere "seeing what is not there" into discrete and testable phenomena. Much work has gone into creating scales for systematically assessing the experience of altered states of consciousness (Dittrich, 1998). Although "hallucinogen" still remains a generic descriptor of a broad class of compounds, it is used herein to describe compounds with a particular combination of effects on mental processes and conscious states. A detailed analysis of anecdotal reports of serotonergic hallucinogen usage reveals that spiritual experiences, dissociation, and well-defined illusions or entities are typically limited to experiences of higher dosages (http://erowid.org/experiences). The average hallucinogenic experience is better understood as an amalgam of, but not limited to: (1) phantasmagoria, or the blending of reality and fantasy; (2) pareidolia, or recognition of patterns in vague or random stimuli; and (3) apophenia, or sensing of patterns or connections in otherwise meaningless or random data. These effects,

respectively, lead to testable hypotheses that the effects of hallucinogens are a gestalt that, at minimum, are mediated through a "wandering" or disruption of normal cognitive processes: (1) the distortion of sensory input and/or lowering the threshold of signal:noise filters; (2) the semantic processing and/or attribution of known shapes/meaning to noise, distorted sensory or emotional data; and (3) misattribution of significance to semantic recognition and connections of distorted thoughts, sensory data, or noise. These specific effects are of particular interest as they reflect the utility of hallucinogens as research tools into the nature of consciousness itself.

1.2. The biochemical basis of hallucinogen pharmacology

Historically, users of hallucinogens believed that the natural sources of these compounds contained spirits or other magical properties that fused with the spirit of the taker, lifting the veil between reality and the world of the spirits. Although this metaphor is illustrative and not altogether incorrect, it is insufficient as an explanation of causality. This idea is akin to the belief of alchemists that the quintessence of the planets as their rays penetrate the earth can transmute base metals, *e.g.* lead, to more desirable metals, such as gold. Much of alchemy was directed at the isolation of this "fifth element." Although quite unsuccessful in this venture, the methods and observations of alchemy laid the foundation for the field of chemistry as we know it today. Although I do not seek to denigrate the spiritual uses of these compounds, modern science prefers a more mechanistic approach to the actions and effects of hallucinogens.

In 1943, five years after he initially synthesized it, Dr. Albert Hofmann made the serendipitous discovery that the semi-synthetic ergot derivative LSD was a potent mind altering substance (Hofmann, 1979). Five years later, a novel hormone was discovered in cow blood serum and named "serotonin," reflecting the fact that it affected vascular tone (Rapport *et al.*, 1948). Soon after, serotonin was identified chemically to be 5-hydroxytryptamine (5-HT) and confirmed to exist in very high concentration in blood platelets (Freyburger *et al.*, 1952; Zucker *et al.*, 1954). Just five years after its initial discovery in blood serum, relatively high concentrations of 5-HT were also discovered in brain tissue (Twarog and Page, 1953), although it was many years later before there was

sufficient experimental support for 5-HT to be considered a central nervous system (CNS) neurotransmitter (Dählstrom and Fuxe, 1964). Soon after came a number of hypotheses that the effects of LSD might arise from the blockage (antagonism) or activation (agonism) of 5-HT receptors in the brain (Gaddum and Hameed, 1954; Woolley and Shaw, 1954; Shaw and Woolley, 1956; Anden *et al.*, 1968).

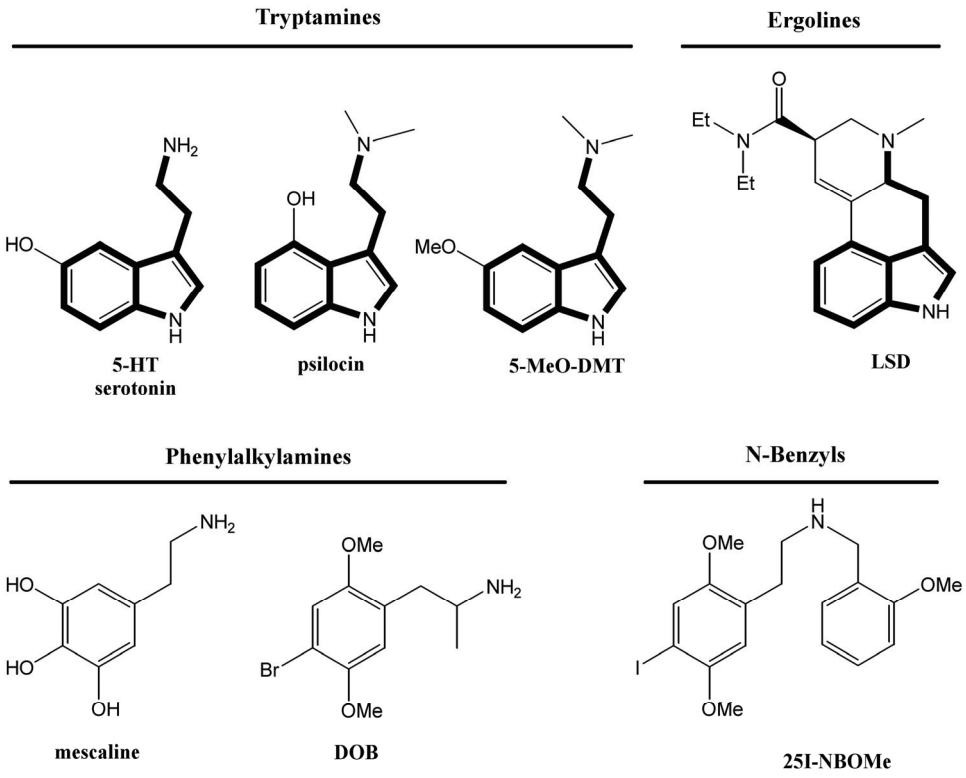


Figure 1.1 Classic and novel hallucinogen classes. Relationship of backbone structure of example compounds to serotonin is indicated in bold.

These hypotheses were partially based on the fact that many of the hallucinogens at the time, particularly the tryptamines, had a chemical structure similar to 5-HT. A comparison of some classic and novel hallucinogen classes/compounds as they relate to 5-HT are illustrated in Figure 1.1. Even LSD, an ergoline, retains the tryptamine scaffold in its tetracyclic structure. Mescaline and the phenylalkylamines based on its structure share few structural features with the tryptamines. In clinical studies of mescaline,

however, it was found to have similar effects and generate cross tolerance to LSD (Balestrieri and Fontanari, 1959), indicating that all three chemical classes, the tryptamines, ergolines, and phenethylamines, may share a similar mechanism of action.

The hypothesis that these compounds antagonize 5-HT receptors in the CNS as their primary mechanism for hallucinogenic action did not last long. As with LSD, BOL, the 2-bromo derivative of LSD, could block the action of serotonin in peripheral tissues (Woolley and Shaw, 1954). BOL, however, is not hallucinogenic (Cerletti and Rothlin, 1955; Rothlin, 1957) and in fact can block the hallucinogenic effects of LSD if co-administered (Ginzel and Mayer-Gross, 1956). Further studies of LSD analogues with different amide substitutions found that LSD-like activity was not correlated with 5-HT antagonist properties (Gogerty and Dille, 1957; Votava *et al.*, 1958).

Initial studies of the agonist character of hallucinogens on CNS 5-HT systems came after the identification that 5-HT was predominantly localized in the raphe nuclei of the brainstem (Dählstrom and Fuxe, 1964). Subsequent studies found that LSD could inhibit the firing activity of the cells in the dorsal raphe nucleus (Aghajanian *et al.*, 1968) and that this action was likely due to pre-synaptic receptors on the dorsal raphe nuclei cell bodies (Aghajanian *et al.*, 1972). Simple hallucinogenic tryptamines, namely DMT and psilocin, similarly were found to suppress the firing of dorsal raphe 5-HT cells (Aghajanian and Haigler, 1975). Even though this was an inhibitory effect, the actions of these drugs were still anticipated to be due to the direct activation of pre-synaptic serotonin receptors on the cell bodies of raphe nucleus cells. Activation of inhibitory serotonin receptors in the raphe nucleus could affect the serotonergic signaling to other brain regions. This hypothesis was attractive as the cell bodies of the raphe nucleus project afferents into several parts of the forebrain (Moore *et al.*, 1978), regions of the brain believed to be responsible for “higher” brain functions. The hypothesis was flawed, however, as the non-hallucinogenic ergoline lisuride also suppresses the firing of dorsal raphe cells (Rogawski and Aghajanian, 1979), whereas mescaline and other hallucinogenic phenethylamines do not directly suppress dorsal raphe cell firing (Aghajanian *et al.*, 1970; Haigler and Aghajanian, 1973).

Only after the cloning of specific serotonin subtype receptors and the development of radiolabeled competition binding assays was there insight into why non-hallucinogens and only some hallucinogens directly affected the firing of cells in the raphe nucleus. Dorsal raphe cells were found to have high expression of 5-HT_{1A} receptors, but not other 5-HT receptor subtypes known at the time (Pazos *et al.*, 1985; Pazos and Palacios, 1985). Activity at 5-HT_{1A} receptors, which phenethylamines lack but tryptamines and ergolines possess, was thus likely responsible for the direct action of both hallucinogenic and non-hallucinogenic agents on the firing of dorsal raphe 5-HT cells (Sprouse and Aghajanian, 1987; Sprouse and Aghajanian, 1988).

Subsequent characterization of the different hallucinogen classes and newly cloned serotonin subtype receptors revealed that the only receptor where all these compounds shared potent activity were 5-HT_2 receptors, particularly the later identified 5-HT_{2A} subtype (see Appendix). Indeed, potential hallucinogenic activity, as measured by drug discrimination studies in rats, could be blocked by the co-administration of a 5-HT_2 antagonist (Glennon *et al.*, 1983), and potency in humans was strongly correlated to 5-HT_2 receptor affinity (Glennon *et al.*, 1984; Titeler *et al.*, 1988). Much work has gone into differentiating the importance of the 5-HT_{2A} or 5-HT_{2C} subtype contributions to hallucinogenic activity (see Nichols, 2004). A general consensus exists, however, that activity at 5-HT_{2A} receptors is essential for hallucinogenic action (McKenna and Saavedra, 1987; Titeler *et al.*, 1988; Pierce and Peroutka, 1989; Sadzot *et al.*, 1989; Branchek *et al.*, 1990; Nichols, 1997; Egan *et al.*, 1998; Krebs-Thomson *et al.*, 1998; Smith *et al.*, 1998; Aghajanian and Marek, 1999; Nelson *et al.*, 1999; Smith *et al.*, 1999; Scruggs *et al.*, 2000; Ebersole and Sealfon, 2002; Nichols, 2004; Braden *et al.*, 2006; Parrish, 2006; Gonzalez-Maeso *et al.*, 2007). The most compelling and recent direct evidence for the necessity of 5-HT_{2A} receptor activation in the action of hallucinogens in humans is that pre-treatment with the relatively selective 5-HT_{2A} receptor antagonists ketanserin or ritanserin can block the hallucinogenic effects of psilocybin, a prodrug for the more active compound psilocin (Vollenweider *et al.*, 1998). This finding supports previous reports that 5HT_2 receptors are primarily located in forebrain areas (Pazos *et al.*,

1985; Pazos *et al.*, 1987) and that stimulation of 5-HT_{2A} receptors by psilocin causes increased activity in these areas as well (Vollenweider *et al.*, 1997).

In order to understand how hallucinogens work at the molecular level, we must have an appreciation of how these molecules bind to and stabilize the active state(s) of the 5-HT_{2A} receptor, how the receptor activation "signal" is transduced down to intracellular effectors, which second messenger pathways are affected by receptor activation, and the relative levels and functional significance of the second messenger molecules produced. Although some functional data are used herein to support the structural features being investigated, a comprehensive review and analyses of the signaling aspects of the 5-HT_{2A} receptor are beyond the scope of this work. Several reviews (Aghajanian and Marek, 1999; Nichols, 2004), recent articles (Kurrasch-Orbaugh *et al.*, 2003a; Parrish and Nichols, 2006; Gonzalez-Maeso *et al.*, 2007), and a complementary thesis to this work by my colleague Jason Parrish (2006) are better suited to address these topics. This work is primarily focused on the physical nature of the ligand-receptor interaction and how molecular models can be used to guide research in these areas.

1.3. GPCR structure and function

G-Protein coupled receptors (GPCRs) are lipid bilayer-spanning proteins, with a transmembrane (TM) domain comprised primarily of seven α-helices. GPCRs are sensor proteins that transduce the signal triggered by extracellular stimuli across the membrane barrier to intracellular signaling cascades. The non-TM helices of GPCRs can exist in random, helical, or β-sheet conformations and generally exist in aqueous environments: the N-terminus connects to TM1 in the extracellular space; the C-terminus connects to TM7 in the intracellular space and usually associates with the membrane through palmitylation; three extracellular loops connect the TM helices in the extracellular space; and three intracellular loop segments connect the TM helices in the cytosolic space. The heptahelical protein receptor superfamily is the fourth largest in the human genome (Venter *et al.*, 2001) with GPCR genes comprising nearly 2% of the entire genome. Approximately 40-50% of the drugs used clinically in humans intentionally target a

GPCR (Teller *et al.*, 2001; Wise *et al.*, 2002; Massotte and Kieffer, 2005), as well as nearly 60-70% of drugs in development (Lundstrom, 2005). GPCR intracellular signaling cascades are mediated primarily by heterotrimeric GTP-binding proteins (G-proteins). The activated G-protein(s) exchange(s) GDP for GTP and the *alpha* subunit dissociates from the *beta-gamma* subunits, which stay associated with the membrane. These subunits can activate various independent signaling cascade pathways.

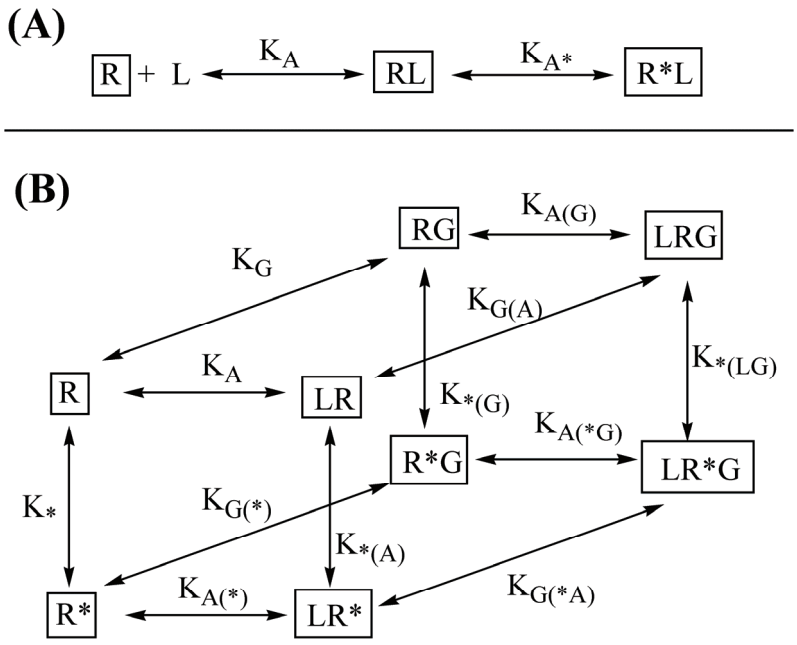


Figure 1.2 Two models of receptor occupancy/activation: **(A)**, the bipartite (two-state) model, and **(B)**, the cubic ternary complex. R is the inactive receptor, L is the ligand, G is a G-protein, R* is the active receptor, and K labels are equilibrium constants.

In no way should it be construed that the GPCR activation process is a linear sequence of events triggered by agonist ligand binding. This process is complicated by the dynamic nature of the protein conformational state. A bipartite model of receptor activation, with only the equilibrium processes of ligand binding and active receptor state taken into account (Figure 1.2A), is sufficient in most cases to explain the binding affinity and functional potency of ligands. This model, however, is often insufficient for explaining aspects of receptor activation such as differences in the robustness of the signal generated (functional efficacy), basal activity of the receptor in the absence of any

ligand, or coupling to effector proteins. Improved models, such as the cubic ternary complex (Figure 1.2B), take into account multiple combinations of receptor, ligand, and effectors to yield "degenerate" active and inactive states of the receptor (De Lean *et al.*, 1980). Even this model continues to be modified and have factors added to it to better fit the empirical data (Samama *et al.*, 1993; Roth *et al.*, 1997a; Egan *et al.*, 2000). The important thing to keep in mind is that a receptor in its natural environment is dynamically and continuously changing conformation and these degenerate states are not always of identical physical structure/conformation, but are of similar energy and/or effect. Agonist activity at a receptor thus becomes the favoring of a statistical distribution of degenerate active states for a particular effector pathway over a given period of time (Weiss *et al.*, 1996).

Due to the membrane-bound nature of GPCRs, it is difficult to crystallize them for X-ray crystallography to determine an absolute structure of any of these states. As discussed in the next section, computational modeling of GPCRs can fill this gap to some degree, but is not without its limitations. Instead, validation of these models and inferred structural features of the receptors typically come from the coordinated replacement of ligand and amino acid side chain chemical groups, namely site-directed mutagenesis, solvent accessibility, and/or cross-linking studies (Roth *et al.*, 1998; Kristiansen, 2004; Pogozheva *et al.*, 2005; Bywater, 2005; Patny *et al.*, 2006). Because of the indirect nature of these experiments, and as Figure 1.2 above helps to illustrate, we must therefore be very careful in extrapolating the data from these experiments to infer the structure of the receptor, for the equilibrium values we empirically derive may not be reflective of a discrete equilibrium step but a continuum of several equilibria. It can often be difficult to clearly attribute the effects of a mutation to a disruption of binding site residue interactions *vs.* disruption of an allosteric regulation site or pathway of signal communication (Süel *et al.*, 2003; Pogozheva *et al.*, 2005). Furthermore, a single amino acid change can in some cases drastically perturb global protein structure. We can reduce these concerns, however, by utilizing many structurally related compounds, comparing different drug classes simultaneously, and comparing the effects of mutating the cognate residues in related receptors.

Table 1.1 Naming scheme for GPCR amino acid residues .

TM	Highest Conserved Residue	Residue Number in h5-HT_{2A}R	Residue Identifier in h5-HT_{2A}R
1	Asn	93	Asn1.50(93)
2	Asp	120	Asp2.50(120)
3	Arg	172	Arg3.50(172)
4	Trp	200	Trp4.50(200)
5	Pro	246	Pro5.50(246)
6	Pro	338	Pro6.50(338)
7	Pro	377	Pro7.50(377)

The 5-HT_{2A} receptor is a monoamine-binding receptor of the rhodopsin-like (Class A) family of GPCRs, the largest sub-family with approximately 90% of all 7-TM receptor proteins as members (Palczewski *et al.*, 2000). Even with low similarity of primary amino acid sequence identity and drastically different molecular structures of agonist ligands, Class A GPCRs are believed to be structurally homologous proteins, that is, they share a high degree of similarity in their 3-D structure and positioning/orientation of particular conserved amino acid residues (Lesk and Chothia, 1986), particularly in their TM helices. To aid in comparing the similarities and differences of amino acid residues in the same position across several homologous receptors with different sequence alignment and numbering, a system of identifying residues beyond their amino acid type and absolute sequence position is necessary. Several attempts have been made to develop such a numbering scheme, with different benefits and drawbacks (Hibert *et al.*, 1991; Oliveira *et al.*, 1993). The scheme developed by Ballesteros and Weinstein (1995), however, has proven the most useful as it is not so reliant as the other schemes on arbitrary definitions of the start of TM helices, use of non-Arabic numbering, or complex relational algorithms. Instead, this method identifies a residue in relation to the most conserved residue in that TM helix, placing its actual sequence identifier in parentheses (Table 1.1). For example, Proline 246 corresponds to the most highly conserved TM5 residue in all Type A GPCRs. Thus in 5-HT_{2A} receptors the identifier for it would be Pro5.50(246); Serine 242 would be identified as Ser5.46(242).

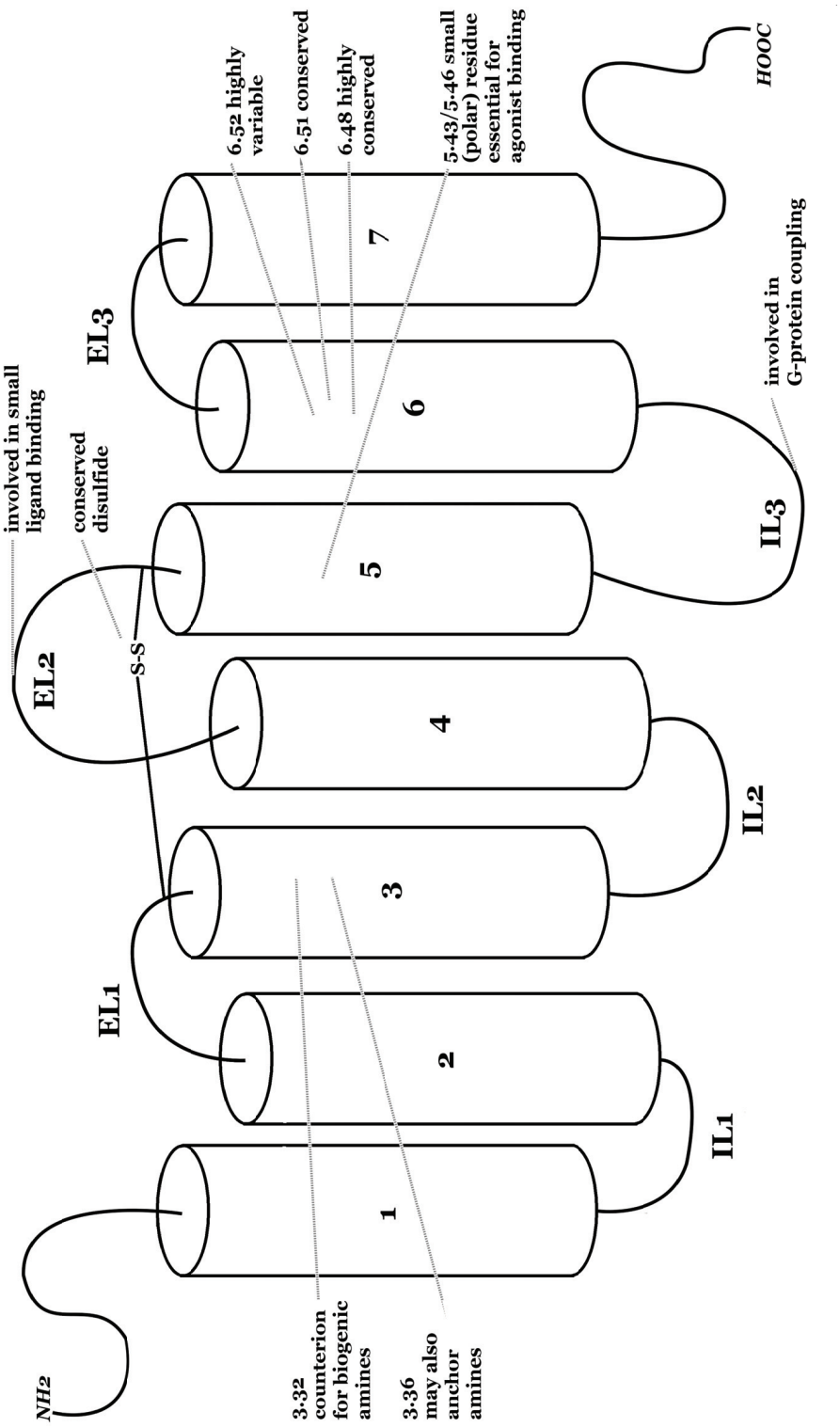


Figure 1.3 Cartoon illustration of GPCR structure. Structural features relevant to 5-HT receptors are indicated.

Figure 1.3 illustrates a generalized cartoon structure of GPCRs. There is a general consensus that the putative binding site for small agonists of Class A GPCRs lies within a solvent-accessible region of the heptahelical bundle of TM helices. A network of evolutionarily conserved residues in all TM helices may mediate the signaling process from the binding site to allosteric sites within the cell (Süel *et al.*, 2003), but primary contacts between the ligand and residues are believed to be in TM helices 3, 5, and 6 (van Rhee and Jacobsen, 1996; Roth *et al.*, 1998; Kristiansen, 2004).

TM3 contains the highly conserved DRY motif, implicated in the biophysical activation mechanism of several GPCRs (Rasmussen *et al.*, 1999; Ghanouni *et al.*, 2001; Weinstein, 2005). For monoamine-binding GPCRs, an acidic residue at position 3.32, Asp3.32(155) in 5-HT_{2A} receptors, is highly conserved. There is ample evidence to demonstrate that this residue is essential in acting as a counterion for the positively charged amine at physiological pH; mutation of this residue to a non-anionic residue results in the most dramatic losses in aminergic agonist and antagonist binding affinities (Ho *et al.*, 1992; Mansour *et al.*, 1992; Wang *et al.*, 1993; Kristiansen *et al.*, 2000). Additional polar contacts in TM3 are highly variable among biogenic amine GPCRs. In 5-HT_{2A} receptors, mutation of Ser3.36(159) showed differential effects on tryptamine and ergoline ligands, depending on the amine substitution. This residue was proposed to be involved in anchoring the protonated amine, and possibly in affecting the efficacies of agonists

The sequence identity of TM5 is the least conserved across all GPCRs (Bywater, 2005). The only highly conserved feature of TM5 is a proline at position 5.50. This high variability likely allows for ligand selectivity across the GPCR family. Most biogenic amine neurotransmitter receptors have polar residues in positions 5.41, 5.42, and/or 5.46. These residues are believed to provide polar contacts for ligands, as is the case with the catechol moiety of dopamine/norepinephrine (Strader *et al.*, 1989; Wang *et al.*, 1991; Pollock *et al.*, 1992; Mansour *et al.*, 1992). There is no evidence indicating Gly5.42(238) in the 5-HT_{2A} receptor interacts directly with ligands for this receptor. Ser5.43(239) in both human and rat 5-HT_{2A} receptors, and Ser5.46(242) in the human 5-HT_{2A} receptor, have been shown to possibly be involved in contacts with tryptamines (Kao *et al.*, 1992;

Johnson *et al.*, 1993; Johnson *et al.*, 1994; Johnson *et al.*, 1997; Shapiro *et al.*, 2000). There is some disagreement, however, between these previous studies as to how tryptamines orient in the binding site (see section 4.2). Phenylalanine residues in TM5, namely Phe5.44(240), Phe5.47(243), and Phe5.48(244), have been implicated in anchoring the aromatic moiety of 5-HT receptor ligands, although without experimental support (Hibert *et al.*, 1991; Edvardsen *et al.*, 1992; Moereels and Janssen, 1993; Westkaemper and Glennon, 1993; Zhang and Weinstein, 1993; Weinstein and Zhang, 1995; Kristiansen and Dahl, 1996). (Weinstein and Zhang, 1995; Almaula *et al.*, 1996). In rhodopsin, a zinc binding site near residues 3.37 and 5.46 may be critical for the structure and function of this receptor (Stojanovic *et al.*, 2004).

Movement of TM6 appears critical for the activity of GPCRs (Gether and Kobilka, 1998; Spalding *et al.*, 1998; Rasmussen *et al.*, 1999; Ghanouni *et al.*, 2001). In 5-HT receptors, agonist contact with TM6 appears to be primarily with a highly conserved tryptophan at position 6.48 (Hibert *et al.*, 1991; Wess *et al.*, 1993) and a phenylalanine residue at position 6.52 that is conserved among 5-HT receptors but not highly in other GPCRs (Choudhary *et al.*, 1993; Choudhary *et al.*, 1995). Ligand contact with a highly conserved phenylalanine residue at position 6.51 in 5-HT receptors appears limited to antagonists (Roth *et al.*, 1997b). In a cluster analysis of statistical coupling in the GPCR family, residues corresponding to Phe5.47(243), Asp3.32(155), Phe6.52(340) and Trp6.48(336) in the 5-HT_{2A} receptor show a similar sensitivity to evolutionary changes of individual amino acids, whereas perturbations of residues corresponding to Phe6.51(339) and Phe6.52(340) are strongly correlated to each other across all GPCRs (Süel *et al.*, 2003). This finding indicates that these residues may be involved in an evolutionarily conserved network of residues important for GPCR signal transduction. Indeed, in 5-HT_{2A} and adrenergic receptors, the cluster of F6.44, W6.48, F6.51 and F6.52 are believed to face the agonist binding site and be components of a key “toggle switch” in the receptor activation process that links binding site accommodation, global receptor conformational change, and allosteric activation of effector signaling (Shi *et al.*, 2002; Weinstein, 2005).

Generally, most of the loop domains of GPCRs are not considered to be directly involved in ligand binding. There is some evidence, however, to indicate that the second extracellular loop (EL2) connecting TM4 and TM5 may be involved in the binding and function of small agonist ligands (Olah *et al.*, 1994; Audoly and Breyer, 1997; Ott *et al.*, 2002; Seong *et al.*, 2003; Shi and Javitch, 2004; Kukkonen *et al.*, 2004). Although the third intracellular loop (IL3) connecting TM5 and TM6 is not believed to be directly involved in ligand binding, there is ample evidence to support the role of IL3 in the selective coupling of the receptors to G-proteins (Strader *et al.*, 1987; Kubo *et al.*, 1988; Kosugi *et al.*, 1992; Samama *et al.*, 1993; Blount and Krause, 1993; McAllister *et al.*, 1993; Shapiro *et al.*, 1993; Tsukaguchi *et al.*, 1993; Oksenberg *et al.*, 1995; Hill-Eubanks *et al.*, 1996). The 5-HT_{2A} receptor is believed to couple primarily to heterotrimeric $G_{q/11}$ proteins to stimulate the PI-PLC-mediated conversion of the membrane lipid phosphatidylinositol to diacylglycerol and inositol triphosphate (Figure 1.4), leading to increased intracellular calcium levels (Conn and Sanders-Bush, 1984; Conn and Sanders-Bush, 1985; Conn and Sanders-Bush, 1986; Barnes and Sharp, 1999). There is additional evidence to show, however, that the signaling of the 5-HT_{2A} receptor may involve other G-proteins, namely $G_{i/o}$ (Raymond *et al.*, 2001; Kurrasch-Orbaugh *et al.*, 2003a; Gonzalez-Maeso *et al.*, 2007), and a different signal may be more relevant to the action of hallucinogens, namely the release of eicosanoid signaling molecules (Parrish, 2006; Parrish and Nichols, 2006). It is anticipated that a structural appreciation of the ligand-receptor interaction may aid in the development of ligands that selectively activate specific second messenger pathways to elucidate further these differences.

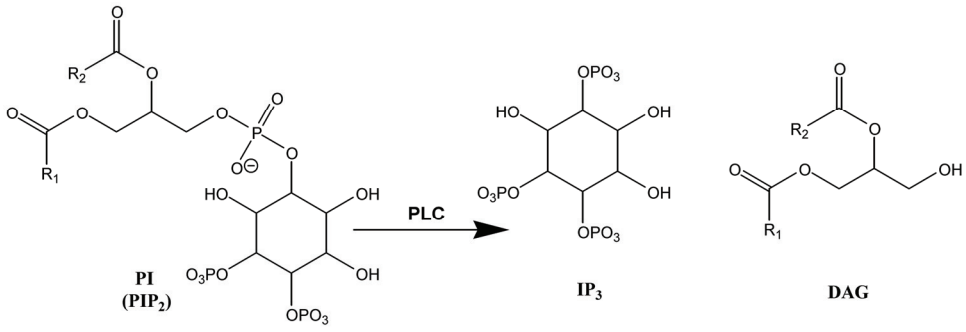


Figure 1.4 PLC-catalyzed hydrolysis of phosphatidylinositol (PI or PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Adapted from Parrish, 2006.

1.4. Development of a h5-HT_{2A} homology model

In the absence of precise structural information for a protein of interest, computer models can provide a convenient means to investigate numerous aspects of the structure/activity relationships (SAR) of ligand-receptor complexes (Pogozheva *et al.*, 2005): (1) provide supportive explanations for pharmacological data from empirical experiments; (2) propose mechanisms of receptor selectivity of ligands and agonist or antagonist properties at a receptor; (3) explore the mechanisms of functional selectivity/agonist-directed trafficking at a receptor; and (4) support the design of potent pharmaceutical agents. As mentioned in the previous section, the homology of the GPCRs allows insights from previous studies of other Class A GPCRs that yield structure prediction, mutagenesis data, spectral information, computer models, and crystal structures to extend to the structure of 5-HT receptors (van Rhee and Jacobsen, 1996; Lu *et al.*, 2002; Kristiansen, 2004; Hillisch *et al.*, 2004; Patny *et al.*, 2006). Similarly, further elucidation of the structure of the 5-HT_{2A} receptor binding site may aid investigations of related proteins, *e.g.* dopamine and norepinephrine receptors.

Homology modeling based on high resolution structural information is considered to be one of the most reliable techniques for generating 3-D computer models of related proteins, although the accuracy of these models is highly dependent on the percentage sequence identity between the two proteins, and drops off rapidly at <30% identity (Baker and Sali, 2001). In an evaluation of virtual docking studies to homology models of receptor binding sites compared to experimentally derived data, it was found that when the sequence identity between the template and model protein was greater than 50%, then up to 5-fold more compounds were identified as active than would have been found randomly (Oshiro *et al.*, 2004). The major source of errors in automated homology modeling comes from both low sequence identity and sequence alignment mismatch (Shacham *et al.*, 2001; Eswar *et al.*, 2003; John and Sali, 2003; Pogozheva *et al.*, 2005). Although sequence identity as low as 20% can be used to generate models that support or suggest site-directed mutagenesis studies, virtual screening of small ligands and structure-based drug design should generally be limited to models based on 50% identity or greater (Baker and Sali, 2001; Hillisch *et al.*, 2004; Oshiro *et al.*, 2004).

Before the crystallization of a mammalian GPCR, generation of GPCR homology models from X-ray crystal structures was limited to the 7-TM template of bacteriorhodopsin. This template was not ideal as bacteriorhodopsin is not a GPCR, shares very little sequence identity (<20%) with GPCRs, and is believed to have different helical packing than GPCRs (Henderson and Schertler, 1990; Schertler *et al.*, 1993; Baldwin, 1993; Lanyi, 1995; Unger *et al.*, 1997). Fortunately, one mammalian GPCR was recently crystallized to yield a high resolution X-ray crystal structure, that of a dark-adapted inverse agonist state of bovine rhodopsin (Palczewski *et al.*, 2000; Okada *et al.*, 2004).

Bovine rhodopsin (bRho) is unique among GPCRs because its ligand, retinal, is covalently attached by a Schiff's base linkage to a lysine residue, K7.43(296). Instead of being a sensor with respect to the presence of retinal, rhodopsin is sensitive to the isomerization state of retinal. In the dark, 11-*cis*-retinal acts as an inverse agonist; that is, more than just keeping the receptor from switching to an active state, it also suppresses any basal activity that the receptor might have in the absence of retinal (Okada *et al.*, 2001). When struck by a light photon of sufficient energy, the 11-*cis* bond of retinal is isomerized to *trans*, dramatically altering its conformation. This isomerization triggers the rearrangement of the rhodopsin protein around the all-*trans*-retinal, and this global shift in protein conformation transmits to intracellular loops that associate with G-protein(s).

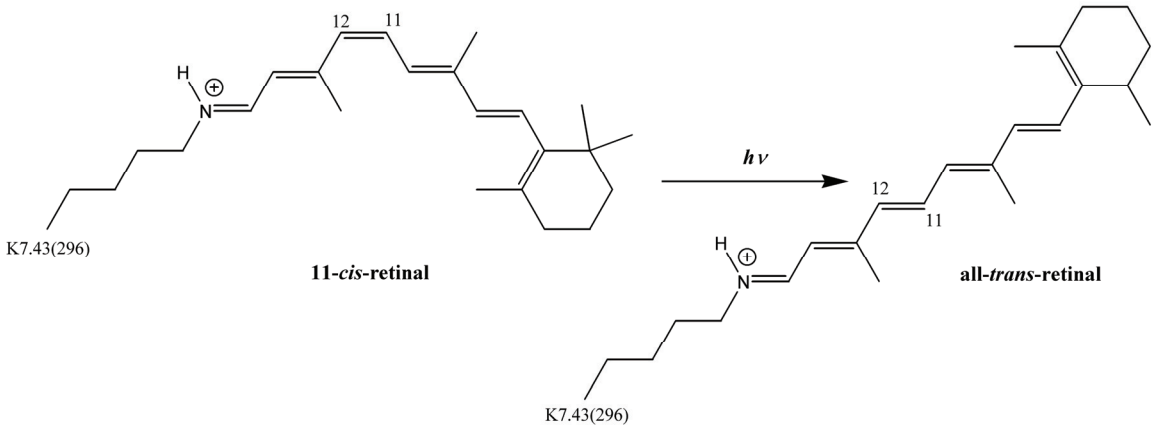


Figure 1.5 Photoisomerization of 11-*cis*-retinal to all-*trans*-retinal in the binding site of bovine rhodopsin (bRho). Adapted from Chambers, 2002.

With some degree of caution, the X-ray crystal structure of bRho is generally believed to be a reasonable and viable template for the construction of homology models of other mammalian GPCRs (Bissantz *et al.*, 2003; Archer *et al.*, 2003; Gouldson *et al.*, 2004; Bywater, 2005; Bosch *et al.*, 2005; Patny *et al.*, 2006). The primary concerns for using the bRho template are twofold: (1) the crystallized form of bRho is in its inactive form, and (2) the low sequence identity between bRho and the wide diversity of other mammalian GPCRs.

It is generally believed that antagonists bind to and/or stabilize the inactive state of the receptor, whereas agonists preferentially bind to and/or stabilize active G-protein-associated active receptor states (Gether and Kobilka, 1998; Kenakin, 2003). As discussed in the previous section, this equilibrium process is dynamic, multi-step, and continuous. Extensive data exist that GPCRs can exist in many conformationally distinguishable states, including many intermediate transition states, and thus a single model for any receptor can never be accurate (Reggio, 2006).Depending on how different the "inactive" and "active" states of the receptor are, it is also quite likely that a different set of receptor residues is involved in interaction with agonists in an active state of the receptors than those involved in binding of ligands to an inactive state (Bywater, 2005). Thus, homology models generated directly from the X-ray crystal bRho template will be suitable only for the study of antagonist binding and inactive states of the receptor. This major limitation was invoked to explain why the bRho template was not viable for constructing a homology model for the CCK_1 GPCR (Archer *et al.*, 2003).

The modeling of a GPCR in an active state of the receptor relies on an understanding of the biophysical events of GPCR activation (Gether and Kobilka, 1998; Bissantz, 2003) and would be much more useful for the study of agonist receptor-ligand interactions (Patny *et al.*, 2006). Unfortunately, modeling the agonist-induced activation process of GPCRs, such as with the commonly used molecular dynamics (MD) simulations, is limited in its accuracy. The time scale for activation has been estimated to be milliseconds for bRho (Arnis *et al.*, 1994) and seconds for the β_2 adrenergic receptor (Ghanouni *et al.*, 2001), much longer than the typical length (10-100 ns) of most MD simulations (Reggio, 2006). Nevertheless, several attempts have been made to generate

3-D models of GPCRs in their agonist-bound active state, such as those for Meta-II (fully active) rhodopsin (Nikiforovich and Marshall, 2003; Slusarz *et al.*, 2006) and β adrenergic receptors (Furse and Lybrand, 2003; Gouldson *et al.*, 2004).

Most relevant to this work is the development of a human 5-HT_{2A} (h5-HT_{2A}) receptor homology model based on an *in silico*-activation of bRho (Chambers and Nichols, 2002; Chambers, 2002). Our laboratory "manually" isomerized the bound 11-*cis*-retinal of bRho to all-*trans*-retinal and allowed the receptor structure to stabilize around this conformationally altered cofactor by utilizing a series of weighted mass MD (Elamrani *et al.*, 1996) and energy minimization simulations that constrained the secondary structure of the TM domain helices as rigid bodies. Distance measurements of key residues correlated well with spin-labeled paired-cysteine mutations in the study of the active state of bRho (Farrens *et al.*, 1996). Furthermore, this h5-HT_{2A} receptor model has proven useful in the design of rigid analogues of phenethylamine agonists for the 5-HT_{2A} receptor (Chambers, 2002; Chambers *et al.*, 2003; McLean *et al.*, 2006a; McLean *et al.*, 2006b).

The other main concern with utilizing the bRho template, whether in an active or inactive form, is the low sequence identity between bRho and the wide diversity of other mammalian GPCRs. A number of prominent deviations from classic α-helical geometry are evident in the structural features of the more conserved TM helices of bRho, namely kinks and/or bends in TM4 and TM6, a short segment of π-helix in TM5, and 3_{10} helix in TM7 (Bywater, 2005; Patny *et al.*, 2006). These deviations from ideal helical structure allow portions of the TM helices to rotate or move relative to the more rigid α-helical segments and are likely involved in conformational changes associated with GPCR activation (Visiers *et al.*, 2000). Homology modeling of bRho based on another template lacking these features would be unlikely to predict structures placing residues in a similar environment as these distortions (Stenkamp *et al.*, 2002; Patny *et al.*, 2006). Moreover, it is not known whether or not these structural features recur in other GPCRs or are unique to bRho (Bywater, 2005).

The h5-HT_{2A} receptor shares only about 16% sequence identity with bRho, although this number goes up to nearly 20% if only the TM helices are examined

(Chambers, 2002). If sequence *similarity* is considered, *e.g.* an aspartate residue in the same position as a glutamate, then homology can be as high as 40-50%. At worst, the spectrum of homology model utility based on sequence *identity* (Baker and Sali, 2001; Hillisch *et al.*, 2004) limits the use of the h5HT$_{2A}$ homology model to directing and/or interpreting site-directed mutagenesis studies. At best, the sequence similarity is just at the edge of being useful for structure-based drug design and small ligand docking. As this model has been somewhat successful in all three areas (Chambers *et al.*, 2003; Parrish *et al.*, 2005; Braden *et al.*, 2006; McLean *et al.*, 2006a; McLean *et al.*, 2006b) it is reasonable to suggest that our h5HT$_{2A}$ receptor model is likely about 90-95% accurate in its main chain (backbone) atom placement (Baker and Sali, 2001).

The natural progression of homology modeling includes phases of model refinement, optimization, and validation (Hillisch *et al.*, 2004; Patny *et al.*, 2006). A common process for model refinement and explanation of pharmacological data is the virtual docking of ligands into the putative binding site of the receptor, followed by energy minimization and MD simulations (Bissantz *et al.*, 2003; Patny *et al.*, 2006). Due to the limitations of desktop computational power, these simulations can be further refined by supplementation of constraints based on site-directed mutagenesis studies.

The ensemble of equations that make up a molecular force field for MD or energy minimization simulations are deterministic analyses of molecular trajectories that mainly rely on classical (Newtonian) descriptions of atomic movement, such as harmonic spring vibration of rigid atomic bodies around a chemical bond or Coulombic attraction of point charges (Xu *et al.*, 2000; Boeyens and Comba, 2001; Scheerschmidt, 2007). MD is distinct from energy minimization as the latter tends to find local energy minima whereas MD simulations attempt to find the global energy minimum by assigning random initial velocities to each atom based on a Boltzmann distribution for a given temperature. This process is followed by iterative integrations of Newton's laws of motion based on the forces calculated from the force field ensemble over 1 femtosecond time steps. Unfortunately, these force fields are limited in their description of non-classical quantum mechanical phenomena (Xu *et al.*, 2000), namely reactions or interactions where bonds are broken or polarized. To reduce computation time, these simulations tend to

parameterize these non-classical processes as classical approximations (Scheerschmidt, 2007), because quantum mechanical calculations are quite computationally expensive.

One of the greatest effects of the parameterization of non-classical effects is on the determination of the energy of hydrogen bonds. In these parameterizations, individual atoms are treated as point charges. A "fudge factor" is added to the calculations of interactions that resemble a hydrogen bond. The calculated energy is weighted favorably for interactions of partial point charges of atoms known to be involved in hydrogen bonds, and with bond angles and distances within experimentally defined ranges. Furthermore, the starting structure for the molecular dynamics simulation (Patny *et al.*, 2006) and lack of proper hydrogen bond identification can have major effects on the quality of the output structures with respect to molecular geometry and macromolecular conformation. For these reasons, one must utilize data from site-directed mutagenesis or other structural studies and be very careful in the selection of aggregates and constraints for these calculations.

In the generation of the human 5-HT_{2A} receptor model based on an activated form of bRho, a number of potential ligand-receptor interactions were identified, most of which are hydrogen bonds (Chambers and Nichols, 2002). It was acknowledged in that work that the h5-HT_{2A} model needs further refinement through an iterative process involving site-directed mutagenesis and generation of rigid analogues of ligands. Indeed, few site-directed mutagenesis studies have been done with h5-HT_{2A} receptors. Moreover, studies of rat 5-HT_{2A} receptors tend to be limited in the compounds utilized or present conflicting opinions of ligand binding orientation and/or binding site structure. Therefore, in the absence of more precise information on active states of neurotransmitter GPCRs, this work presents a number of hypotheses that seek to utilize our h5-HT_{2A} homology model, site-directed mutagenesis, and screening of a large library of several hallucinogen classes in order to optimize and validate the topology of the 5-HT_{2A} receptor agonist binding site.

CHAPTER 2. SPECIFIC AIMS

2.1. Rationale

In the development of our h5-HT_{2A} homology model and studies of its subsequent utility a number of implied interactions were assumed and used for either qualitative support of the model or the pharmacological data gathered (Chambers and Nichols, 2002; Chambers *et al.*, 2003; Parrish *et al.*, 2005; McLean *et al.*, 2006a). Upon closer inspection, it became apparent that there was weak or insufficient empirical evidence to confirm many of these interactions in the h5-HT_{2A} receptor and that the model itself needed validation and optimization in order to proceed with its use in directing ligand synthesis. The proposed interaction of the protonated amine of the ligands and Asp3.32(155) is strongly supported across a number of biogenic amine GPCRs (Strader *et al.*, 1988; Wang *et al.*, 1993; Kristiansen *et al.*, 2000) and thus was not further investigated. As detailed below, previous investigations of the interactions with other residues in the 5-HT_{2A} receptor binding site were limited to particular compounds/classes, provided conflicting conclusions, or were never performed. The following hypotheses were thus set forth in order to: (1) verify and expand previous investigations of 5-HT_{2A} receptors; (2) investigate novel ligand-receptor interactions; and (3) support the topology of the binding site as defined by virtual docking simulations to our h5-HT_{2A} receptor homology model. Modeling techniques cannot be used solely to prove the fidelity of a model, but the model can be used as a hypothesis generator to provide opportunities not only to direct ligand synthesis but to verify the model itself.

2.2. Hypotheses

2.2.1. Specific Aim 1

Utilization of the h5-HT_{2A} homology model to provide a qualitative explanation for the empirical observation that phenylisopropylamine agonists are more efficacious than their phenethylamine homologues at stimulation of the 5-HT_{2A} receptor-coupled PLC-mediated second messenger pathway. Experiments were undertaken to provide pharmacological support for limited human anecdotal data as to the relative potencies of phenylalkylamines, depending on the presence of an alpha-methyl substituent and the stereochemistry of the resulting phenylisopropylamines. In addition to the collection of binding and functional data, virtual docking simulations to our h5-HT_{2A} receptor homology model were performed and the possible effects of the differential receptor accommodation that was observed are discussed.

2.2.2. Specific Aim 2

To test the hypothesis that specific aromatic-aromatic and hydrogen bond interactions with residues in TM6 of the h5-HT_{2A} receptor predicted from virtual docking simulations were involved in the activity of classic and novel agonist ligands. A new series of super-potent 5-HT_{2A} agonists was characterized and provided a useful tool for investigating the aromatic ligand-receptor interactions of Phe6.51(339) and Phe6.52(340) in the 5-HT_{2A} receptor binding site, as well as identifying a novel role for Phe6.51(339). This new class of compounds, along with certain ergolines, was also investigated for a predicted hydrogen bond interaction with Asn6.55(343) in TM6. Effects of mutating these receptor residues on ligand binding and ligand-induced receptor activation are discussed.

2.2.3. Specific Aim 3

To test the hypothesis that specific hydrogen bond interactions, predicted by virtual docking simulations of tryptamines, occur between the ligands and polar residues in TM5 of the h5-HT_{2A} receptor. Virtual docking experiments of ring-substituted tryptamines resulted in docking orientations that indicated a hydrogen bond between: Ser5.43(239) and the 4- or 5-oxygen of the ligand; and Ser5.46(242) and the N(1) indole nitrogen of the tryptamine ligand. Effects resulting from mutation of these receptor residues, as well as complementary changes of ligand substitution, are discussed.

2.2.4. Specific Aim 4

To test the hypothesis observed in virtual docking simulations of phenylalkylamines that this ligand class interacts with residues in TM5 of the h5-HT_{2A} receptor, including a key hydrogen bond interaction. Virtual docking experiments with ring-substituted phenylalkylamines resulted in docking orientations that indicated a hydrogen bond between Ser5.43(239) and the 5-oxygen of the ligands. Virtual docking orientations indicated that no hydrogen bond could be formed between phenylalkylamine ligands and Ser5.46(242). These orientations also placed the ligand alkyl or halogen 4-substituent in proximity to Gly5.42(238). Effects resulting from mutation of these residues, as well as complementary changes of ligand substitution, are discussed.

2.2.5. Specific Aim 5

To test the hypothesis, predicted from virtual docking experiments, of hydrogen bonding between the 2-methoxy of phenylalkylamine ligands and polar residues in TM3 of the h5-HT_{2A} receptor. Virtual docking experiments of ring-substituted phenylalkylamines resulted in docking orientations that indicated a hydrogen bond between both Ser3.36(159) and Thr3.37(160) and the 2-oxygen. Effects resulting from mutation of these residues, as well as complementary changes of ligand substitution, are discussed.

2.3. Significance

The serotonergic hallucinogens remain unique tools for investigating aspects of normal consciousness, and as well may represent novel therapeutic agents for several psychiatric and medical indications, such as obsessive compulsive disorder, long-term pain management, and improving quality at the end-of-life. Slight changes in ligand structure can have subtle to dramatic effects on the subjective experience, potency, and duration of these compounds. A fundamental understanding of the structural requirements of both the ligand and receptor for binding to and activation of the receptor may aid not only in the development of more potent and receptor-selective agents, but in identifying functionally selective agents as well that favor one particular intracellular signaling system over another. By reducing the action at other receptors or enhancing signaling through a particular second messenger system, we may be able to create more effective research tools and chemotherapeutic agents with specific effects, while reducing deleterious side effects.

In the realm of medicinal chemistry, ligand synthesis is slowly evolving away from massive ligand library synthesis and systematic screening of hundreds or even thousands of compounds in search of good "hits" (Bajorath, 2001; Hillisch *et al.*, 2004). As computational power increases and computational expense for more elaborate equations, algorithms, and virtual simulations declines, the ability to carry out computer-aided rational drug design becomes more feasible, even at the desktop level. The time and cost benefits of limiting synthetic library generation and testing at the design level can be enormous. As computational power increases, the simulations will become not only faster but more accurate as more factors dropped from earlier approximations to save time are restored. A model system, however, is inherently limited in its accuracy, will never reach full fidelity, and must be extensively validated and supported by empirical data. With these concerns in mind, this work is directed toward the validation of our h5-HT_{2A} receptor homology model. Moreover, because the h5-HT_{2A} receptor is a member of a large family of homologous GPCRs, the structural insights gained in these investigations can be extended to multiple other receptor systems, dopamine and norepinephrine for example.

CHAPTER 3. MATERIALS AND METHODS

3.1. Chemicals and Supplies

[^{3}H]Ketanserin, [^{125}I]4-iodo-2,5-dimethoxyphenylisopropylamine ([^{125}I]-DOI), and [^{3}H]*myo*-inositol, GF/B Unifilters, and Microscint-O were obtained from Perkin Elmer (Wellesley, MA). Unsupplemented Dulbecco's Modified Eagle Medium (DMEM), G-418, lithium chloride, pargyline, serotonin (5-HT), 5-methoxytryptamine (5-MeO-T), 5-methyltryptamine (5-Me-T) and tryptamine were obtained from Sigma-Aldrich (St. Louis, MO). 5-Methoxy-N(1)-isopropyl-tryptamine (5-MeO-iPrT) and 1-methylserotonin (MeS) were obtained from the NIMH Chemical Synthesis and Drug Supply Program (http://nimh-repository.rti.org). Bovine calf serum and fetal clone serum were obtained from VWR (West Chester, PA) and dialyzed fetal bovine serum from Atlanta Biologicals (Lawrenceville, GA). Unless otherwise indicated, all other cell culture media, chemicals, and antibiotics were obtained from Invitrogen/Gibco (Carlsbad, CA). All restriction and polymerase enzymes were obtained from New England Biolabs (Beverly, MA). All other test ligands used in this study were synthesized in our laboratory using standard methods. The purity and identity of synthesized compounds were verified with TLC, melting point, NMR, mass spectrometry, and elemental analysis. Stock solutions of compounds were prepared as the following salts: 5-HT as the creatine sulfate salt; 5-MeO-DMT, 1-methylserotonin, and psilocin as the maleate salts; 5-methoxy-1-isopropyltryptamine as the oxalate salt; all other compounds were used as their HCl salts.

3.2. Cell Culture Methods

Mouse fibroblast (NIH-3T3) cells stably expressing either the rat 5-HT_{2A} receptor (GF-6; 5500 fmol/mg) or the r5-HT_{2C} receptor (PØ; 7500 fmol/mg) were the kind gift of Dr. David Julius. Chinese hamster ovary (CHO) cells stably expressing the human 5-HT_{1A} receptor (CHO-1A; 500 fmol/mg) were the kind gift of Upjohn. All cell types were maintained at 37 °C with 5% CO_2 with DMEM, pH 7.4, supplemented with 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Complete DMEM for regular growth further contained 5% (v/v) bovine calf serum and 5% (v/v) fetal clone serum ("complete growth DMEM"), whereas media for propagating cells for membrane preparations and PI hydrolysis contained 10% (v/v) dialyzed fetal bovine serum ("complete assay DMEM"). All complete DMEM media for stably expressing cell lines contained 300 μg/mL G-418 for GF-6 and PØ cells, 22.5 units/mL Hygromycin B for CHO-1A cells, and 30 μg/mL Zeocin for Hh2A wild type and mutant cell lines (described below).

3.3. Receptor expression and mutagenesis

3.3.1. Establishing stable wild type human 5-HT_{2A}R cell lines

The h5-HT_{2A} receptor gene insert was excised from pcDNA3.1-h5HT_{2A}R (Guthrie cDNA resource center, Sayre, PA; http://www.cdna.org) with PmeI and XhoI and subcloned into the pBudCE4 vector (Invitrogen), that was cut with NotI, blunted with T4 DNA Polymerase in the presence of dNTPs, and then cut with XhoI. Insert and vector were purified, quantified, ligated, and amplified utilizing standard methods. Orientation, identity, and sequence were verified by primer-directed sequencing (Retrogen, San Diego, CA) utilizing custom synthesized EF-1α forward and BGH reverse primers (Integrated DNA Technologies, Coralville, IA). Human embryonic kidney (HEK-293) cells were allowed to grow to about 90% confluency in a 100 mm cell culture dish and were subsequently transfected with 3.5 μg pBudCE4-h5HT_{2A}R utilizing the Fugene-6 lipofection reagent (Roche Biomolecules, Indianapolis, IN) in a 3:1 (w:v) ratio in

OptiMEM. The following day, the cells were detached using 2.5 μg trypsin, resuspended in 5 mL complete DMEM, and passaged into four 100 mm cell culture plates, each with 0.1, 0.5, 1, or 2 mL of the cell suspension. The media was replaced the next day with complete DMEM supplemented with 60 μg/mL Zeocin and replaced with this selection media every two days until visible colonies formed. Colonies of stable Hh2A clones surviving selection and maintenance with Zeocin were then assayed for $h5HT_{2A}R$ expression by saturation isotherm binding assays. Clones were selected for high ($Hh2A_{hi}$; 8000 fmol/mg) and moderate ($Hh2A_{lo}$; 1600 fmol/mg) expression.

3.3.2. Transient expression of the human 5-HT_{2C} receptor

HEK-293 cells were passaged into 150 mm cell culture plates and allowed to grow in complete assay DMEM to about 90% confluence. Each plate of cells was then transfected with 5.5 μg pcDNA3.1-$h5HT_{2C}R$ (Guthrie cDNA resource center, Sayre, PA; http://www.cdna.org) utilizing the Fugene-6 lipofection reagent (Roche Biomolecules) in a 3:1 (w:v) ratio in OptiMEM. The following day, cells were prepared as membrane preparations as described below.

3.3.3. Point mutations and establishing stable h5-$HT_{2A}R$ mutant cell lines

The $h5HT_{2A}R$ insert was excised from pcDNA3.1-$h5HT_{2A}R$ (Guthrie cDNA resource center, Sayre, PA; http://www.cdna.org) and subcloned into the pLNCX2 vector (Clontech, Mountain View, CA) using HindIII and XhoI. The digestion of pcDNA3.1-$h5HT_{2A}R$ with XhoI was spiked with HindIII for only 15 minutes as there is a HindIII site within the $h5HT_{2A}R$ gene. Insert and vector were purified, quantified, ligated, and amplified utilizing standard methods. Orientation, identity, and sequence were verified by primer-directed sequencing (Retrogen, San Diego, CA) utilizing custom primers provided with the pLNCX2 vector. Site-directed mutagenesis of pLNCX2-$h5HT_{2A}R$ was performed using the QuikChange Kit (Stratagene, La Jolla, CA) method of dual complementary mutant primer PCR followed by DpnI digestion of parental vector DNA. Table 3.1 indicates sense primers utilized (Integrated DNA Technologies) with

nucleotide(s) changed in bold. Mutant inserts verified by primer directed sequencing (Retrogen) were then subcloned into the pBudCE4 vector using BglII and NotI. Orientation, identity, and sequence were verified by primer-directed sequencing utilizing custom synthesized EF-1α forward and BGH reverse primers (Integrated DNA Technologies). HEK-293 cells were transfected, colonies selected, and receptor expression verified as described above.

Table 3.1 Sense primers for site directed mutagenesis. Altered nucleotides are indicated in bold and | delineates the codon. Complementary anti-sense primers were used for PCR as well.

Residue	Mutation	Mutant Sense Primer Sequence
S3.36(159)	S159A	GGACGTGCTCT|TC**G**|CCACGGCCTCCATCATGC
T3.37(160)	T160A	CGTGCTCTTCTCC|**G**CG|GCCTCCATCATGCACC
G5.42(238)	G238A	GATGATAACTTTGTCCTGATC|G**C**C|TCTTTTGTGTCATTTTTC
G5.42(238)	G238V	GATGATAACTTTGTCCTGATC|G**T**C|TCTTTTGTGTCATTTTTC
S5.43(239)	S239A	CTTTGTCCTGATCGGC|**G**CT|TTTGTGTCATTTTTCATTCCC
S5.46(242)	S242A	CCTGATCGGCTCTTTTGTG|**G**CA|TTTTTCATTCCCTTAACC
F6.51(339)	F339L	GGTGATGTGGTGCCCT|TT**G**|TTCATCACAAACATCATGGCCG
F6.52(340)	F340L	GGTGATGTGGTGCCCTTTC|TT**G**|ATCACAAACATCATGGCCG
N6.55(343)	N343A	CCCTTTCTTCATCACA|**GC**C|ATCATGGCCG

3.4. Receptor Binding Assays

3.4.1. Membrane preparations

Cells for membrane preparation were propagated into 150 mm cell culture dishes in complete assay DMEM with appropriate selection antibiotics and allowed to grow at 37 °C under 5% CO_2 to about 90-100% confluency. While on ice, cells were detached using a scraper and transferred to pre-chilled 50 mL centrifuge tubes. Cells were

pelletized by centrifugation at 2,000xg in a tabletop clinical centrifuge at 4 °C for 10 minutes. The supernatant was discarded, the pellets were then resuspended by repeated pipetting in pre-chilled binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA; pH 7.4) and this suspension was then transferred in 1.0 mL aliquots into pre-chilled microcentrifuge tubes. The microcentrifuge tubes were then spun at 15,000xg at 4 °C for 20 minutes, the supernatant was immediately aspirated, and the pellet-containing tubes were stored at -80 °C until needed.

3.4.2. Saturation isotherm binding assays

Saturation isotherm binding assays utilized 0.25-10 nM [^{3}H]ketanserin or 0.125-5 nM (±)-[^{125}I]DOI. Nonspecific binding was defined as the binding measured in the presence of 10 μM of the antagonists ketanserin, for 5-HT_{2A} receptors, or mianserin for 5-HT_{2C} receptors. Previously prepared membrane preparations were resuspended in binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA; pH 7.4) and added to each tube containing binding buffer, radioligand dilution, and either binding buffer (total binding) or cold antagonist (non-specific) to a total volume of 250 μL. Each total and non-specific data point was done in duplicate. Racks of tubes were briefly mixed by vortexing, tapped to move droplets to the bottom of the tubes, and incubated at 25 °C for 45-60 minutes. Assays were terminated by rapid filtration through GF/B Unifilters that had been washed with pre-chilled wash buffer (10 mM Tris, 150 mM NaCl; 4 °C) in a Packard 96-well harvester. Radioligand dilutions were spotted on a separate plate and all plates were either left to dry overnight or dried at 60 °C for 1 hour. 40 μL Microscint-O was added to each well of the dried plates and radioactivity counted using a TopCount microtiter scintillation counter (Perkin Elmer).

3.4.3. Competition binding assays

Ligands were tested in increasing concentrations in competition binding assays for their ability to displace 0.25 nM [^{125}I]DOI at 5-HT_{2A} and 5-HT_{2C} receptors, 0.5 nM [^{3}H]ketanserin at 5-HT_{2A} receptors, and 0.5 nM [^{3}H]8-OH-DPAT at 5-HT_{1A} receptors.

Previously prepared membrane preparations were resuspended in binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA; pH 7.4) and added to each tube containing binding buffer, radioligand dilution, and test compound dilution to a total volume of 500 µL in binding buffer. Each test compound dilution was done in duplicate. Assays were mixed, incubated, terminated, and counted as described above for saturation binding assays.

3.5. Inositol phosphates accumulation functional assays

Compounds were tested for their ability to stimulate hydrolysis of radiolabeled phophatidylinositides (PI) by measurement of radiolabeled inositol phosphates accumulation. Cells were seeded into 24 well cell culture plates and allowed to grow at 37 °C under 5% CO_2 in complete assay DMEM with appropriate selection antibiotic to about 70% confluency. The media of each well was then replaced with CMRL media containing 1 µCi/mL [^{3}H]*myo*-inositol and plates were incubated for 18-24 hours at 37 °C under 5% CO_2. Each well was then pretreated with 10 mM LiCl/10 µM pargyline for 15 minutes at 37 °C, followed by stimulation with increasing concentrations of test drug and incubation at 37 °C for 30 minutes. Each assay plate was normalized to wells stimulated with water (0%) and a concentration of serotonin chosen to be maximally stimulating (100%). For this reason, intrinsic activity for serotonin in all tables and calculations was set to 100%. Assays were terminated by replacing the assay media with 10 mM formic acid and storage at 4 °C for at least 16 hours. Radiolabeled inositol phosphates were separated and collected by ion exchange chromatography on Dowex-1 anion exchange columns. Ecolite (MP Biomedicals, Solon, OH) was added to each scintillation vial, vials were shaken and then counted in a Beckman-Coulter LS6500 liquid scintillation counter (Fullerton, CA).

3.6. Computational modeling

3.6.1. Virtual docking simulations

Ligand structures were drawn and energy minimized (Powell method, 0.01 kcal/mol*A gradient termination, MMFF94s force field (Halgren, 1999a; Halgren, 1999b), MMFF94 charges, 1000 maximum iterations) using the Sybyl modeling program (Tripos, St. Louis, MO). The *in silico*-activated homology model of the h5-HT_{2A} receptor was prepared as previously described (Chambers and Nichols, 2002). Virtual dockings of energy minimized ligands to the h5-HT_{2A} receptor were performed using the program GOLD (Cambridge Crystallographic Data Center, Cambridge, UK) and scored using GOLDScore with default settings typically without constraints. Early docking simulations constrained the GOLDScore fitness algorithm to orientations containing ligand-protein interactions implicated by site-directed mutagenesis and previous modeling (Chambers and Nichols, 2002) to possibly be essential for binding. Distance constraints of 2-3 Å were set between: the side chain carbonyl carbon of D3.32(155) to the amine nitrogen of the ligand (Wang *et al.*, 1993; Kristiansen *et al.*, 2000); the side chain oxygens of S3.36(159) and T3.37(160) to the ligand 2-position oxygen (Almaula *et al.*, 1996); and the side chain oxygen of S5.43(239) to the ligand 5-position oxygen (Johnson *et al.*, 1997; Shapiro *et al.*, 2000). Later docking simulations were performed without any constraints, however, the constraints outlined above were used in subsequent energy simulations, as described below, in order to limit excessive translational motion that might move the ligand out of the binding site. The highest ranked docking output structures were merged with the h5-HT_{2A} receptor model and analyzed with Sybyl.

3.6.2. Energy minimization and molecular dynamics simulations

Merged ligand-receptor structures were energy minimized as a subset based on the ligand molecule (aggregates set to monomers >8Å radius from the ligand, monomers >12Å radius ignored, Powell method, 0.1 kcal/mol* Å gradient termination, MMFF94s force field, MMFF94 charges, distance dielectric of 4, 1000 maximum iterations).

Constraints for subsequent molecular dynamics simulations and minimizations in Sybyl were defined as above for GOLD. Hydrogen bond constraints, however, were defined as either: a distance range constraint of 1-2 Å between each polar residue's hydrogen and the appropriate oxygen atom on the ligand for ligands with only H-bond acceptor oxygens, or 2-3 Å between each polar residue's oxygen and the appropriate oxygen atom on the ligand for ligands with either H-bond donor or acceptor OH groups. Constrained molecular dynamics simulations were then run on the energy-minimized ligand-receptor structures (aggregates, constraints, force field, charges, and dielectric as outlined above, NTV ensemble at 300K, Boltzmann distribution of initial velocities, 5000 steps of 1 fs, and 5 fs snapshots). Structures with lowest potential energy after the first 1000 fs equilibration period were then energy minimized as outlined above with defined constraints.

3.7. Data Analysis

GraphPad Prism Software (GraphPad Software Inc., San Diego, CA) was used to calculate nonlinear regression curves based on the Cheng-Prusoff equation for a one-site model in order to obtain K_i (affinity) values for radioligand displacement, as well as variable slope sigmoidal dose-response curves for EC50 (potency) and intrinsic activity from PI hydrolysis. Variable hill slopes for PI hydrolysis assays typically ranged from 0.6-1.4. This software also was utilized to perform two-way ANOVA on pK_i, pEC50, and intrinsic activity values at human wild type and mutant receptors, with a Bonferroni post-test to compare replicate mean values of multiple mutant receptors to the wild type. Unpaired two-tailed Student's T-tests were performed on pK_i, pEC50, and intrinsic activity values from a single mutant and corresponding wild type values. Values obtained from mutant receptors were considered statistically distinguishable from wild type if the statistical models generated $p<0.05$. Changes in the standard Gibbs free energy of binding ($\Delta\Delta G°$) due to the mutations were calculated from the K_i values at 25 °C with equation 3.1, as follows:

$$\Delta(\Delta G°)\ \Delta G°_{mutant} - \Delta G°_{wild\ type}\ RT \ln(K_{mutant}/K_{wild\ type}) \qquad \text{Eq. 3.1}$$

Changes in EC50 values were transformed to normalize the scale by taking the difference of the $\log_{10}$ value (ΔEC50) with equation 3.2 as follows:

$$\Delta pEC50\ pEC50_{mutant} - pEC50_{WT}\ -logEC50_{mutant} - (-logEC50_{WT}) \quad \text{Eq. 3.2}$$

Changes in intrinsic activity (ΔInt.Act.) were calculated with equation 4 as follows:

$$\Delta Int.Act.\ Int.Act._{mutant} - Int.Act._{WT} \quad \text{Eq. 3.3}$$

Arbitrary thresholds of "weak effects" were defined as changes in $\Delta G°$ of $\leq$ 0.5 kcal/mol or less, pEC50 of approximately 1 log order (10-fold) or less, and changes in intrinsic activity of 25% or less. Virtual docking figures were generated using pyMol (DeLano Scientific, San Carlos, CA; http://www.pymol.org). As discussed in the introduction, amino acid residues are numbered both with their position in the h5-HT_{2A} receptor and their position relative to the most highly conserved residue of that transmembrane segment (Ballesteros and Weinstein, 1995)

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Functional effects of alpha methyl substitution on ring-substituted phenylalkylamines and possible receptor accommodation

The main objective of this specific aim was to provide human 5-HT_{2A} receptor binding data and a qualitative analysis of the results of virtual docking experiments in the context of all the pharmacological data. This project was collaborative and it should be noted that the human functional data and all the rat pharmacological data in this section were generated by Jason Parrish. These results have been published in the Journal of Neurochemistry (Parrish *et al.*, 2005). Additional human 5-HT_{2A} receptor binding data have been added to the tables and Figure 4.1 is reused in fair use.

A series of phenethylamines (PEAs) and their corresponding phenyliso-propylamines (PIAs) were tested for different effects in binding and activation of human and rat 5-HT_{2A} receptors (Figure 4.1). PEA/PIA pairs differed structurally only in the absence or presence of an alpha methyl group (*e.g.* 2CB vs. DOB). Eudismic ratios for binding and functional activity of PIA stereoisomer pairs were also determined (*e.g.* (*R*)-DOB vs. (*S*)-DOB). Compounds were tested for their binding affinity (K_i) at rat (GF6 cells) and human (Hh2A cells) 5-HT_{2A} receptors based on their ability to displace [^{125}I]-DOI. Functional activity of these drugs was compared as well, as measured by ability to stimulate receptor-mediated radiolabeled inositol phosphate release mediated by both human (A20 cells) and rat (GF6 cells) 5-HT_{2A} receptors. Functional activity was reported by relative potency (EC50) and efficacy (intrinsic activity). Representative compounds were docked into the h5-HT_{2A} receptor homology model to examine any differential receptor accommodation that could account for the observed pharmacological data. Compounds used for this specific aim are illustrated in Figure 4.1.

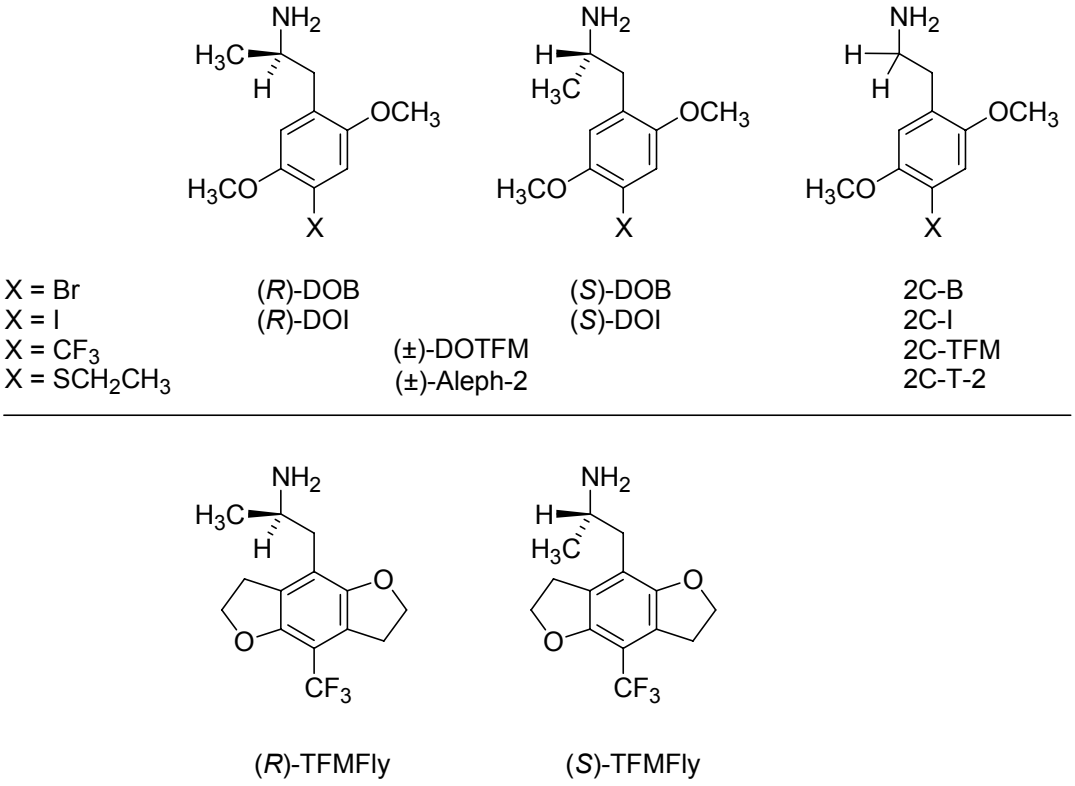


Figure 4.1 Structures of phenylalkylamines used in Specific Aim 1.

4.1.1. Results

The effects of the presence or absence of an alpha methyl group on the binding and activity of phenylalkylamines

As Table 4.1 indicates, with the exception of 2C-TFM vs. DOTFM at human receptors, the PEA/PIA pairs (*e.g.* 2C-B and DOB) had statistically indistinguishable affinities (K_i) for human and rat 5-HT_{2A} receptors. Similar lack of differences was seen in comparisons of functional potencies (EC50). The only statistically discernable differences between nearly all PEA/PIA pairs was in functional efficacy (intrinsic activity).

Table 4.1 Effects of phenylalkylamine alpha-methyl substitution on binding and activity at human and rat 5-HT_{2A} receptors. K_i and EC50 values are presented as the mean and (SEM) in nM from at least three independent experiments. * indicates $p < 0.05$ generated from Student's T-tests comparing K_i, EC50, or intrinsic activity for each PEA/PIA pair.

DRUGS	Hh2A (h5-HT_{2A})	A20 (h5-HT_{2A})		GF-6 (r5-HT_{2A})		
	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)
(±)-DOB	0.68 (0.10)	17.4 (3.0)	70.2 (4.0)	0.66 (0.13)	25.9 (2.1)	87.2 (2.4)
2C-B	0.89 (0.04)	18.2 (2.3)	40.0 (4.5)*	0.66 (0.11)	27.0 (2.0)	64.8 (4.6)*
(±)-DOI	0.64 (0.06)	9.7 (1.8)	49.7 (2.2)	0.65 (0.12)	19.2 (2.6)	77.1 (2.8)
2C-I	0.73 (0.06)	9.8 (1.7)	30.3 (1.5)*	0.65 (0.07)	19.0 (2.6)	59.4 (4.1)*
(±)-DOTFM	0.46 (0.02)	10.1 (1.6)	50.1 (3.7)	0.61 (0.09)	57.0 (2.0)	77.6 (3.8)
2C-TFM	0.69 (0.04)*	7.7 (1.5)	25.7 (1.6)*	0.65 (0.10)	47.6 (5.3)	41.7 (3.4)*
(±)-ALEPH-2	0.70 (0.05)	13.1 (1.6)	57.0 (4.4)	1.78 (0.30)	76.3 (4.3)	84.2 (1.7)
2C-T2	0.78 (0.02)	14.4 (2.1)	43.5 (4.6)	1.81 (0.23)	84.6 (13)	78.7 (2.2)

* $p < 0.05$

The effects of the spatial orientation of the alpha methyl group on the binding and activity of phenylisopropylamines

As Table 4.2 indicates, with the exception of *R/S*-TFMFly, the (*R*)-isomer of all (*R/S*)-isomer pair comparisons had higher affinity and functional potency than the (*S*)-isomer at both human and rat 5-HT_{2A} receptors. This was the case as well for functional efficacy at human 5-HT_{2A} receptors. Functional efficacies for (*S*)-isomers at rat 5-HT_{2A} receptors do appear lower than (*R*)-isomers; these numbers, however, were not statistically discernable from each other.

Table 4.2 Effect of alpha methyl orientation of PIAs on binding and activity at human and rat 5-HT_{2A} receptors. Data are presented as the mean and (SEM) from nonlinear regression fits of a single binding site model for K_i values and normalized variable slope (typically 0.4-1.6) sigmoidal dosage-response curves for estimates of EC50 and intrinsic activity. All data are from at least three independent experiments. * indicates $p < 0.05$ generated from Student's T-tests comparing K_i, EC50, or intrinsic activity for each (*R*)- vs. (*S*)-enantiomer pair.

DRUGS	Hh2A (h5-HT_{2A})	A20 (h5-HT_{2A})		GF-6 (r5-HT_{2A})		
	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)
(*R*)-DOB	0.29 (0.04)	6.5 (1.2)	79.5 (4.5)	0.27 (0.05)	15.3 (2.5)	82.4 (1.2)
(*S*)-DOB	1.86 (0.18)*	25.3 (1.0)*	50.1 (7.9)*	1.49 (0.27)*	74.4 (10.5)*	75.7 (3.1)
(*R*)-DOI	0.27 (0.02)	4.8 (0.3)	59.8 (2.3)	0.31 (0.03)	17.2 (2.9)	74.9 (4.4)
(*S*)-DOI	0.90 (0.04)*	14.7 (0.6)*	38.4 (1.3)*	0.98 (0.17)*	54.2 (7.9)*	62.4 (6.9)
(*R*)-TFMfly	0.12 (0.01)	5.0 (1.0)	73.0 (4.6)	0.15 (0.01)	9.6 (1.1)	79.0 (3.7)
(*S*)-TFMfly	0.49 (0.03)*	8.3 (1.2)	44.6 (1.6)*	0.34 (0.05)*	8.1 (0.4)	70.4 (5.8)

* $p < 0.05$

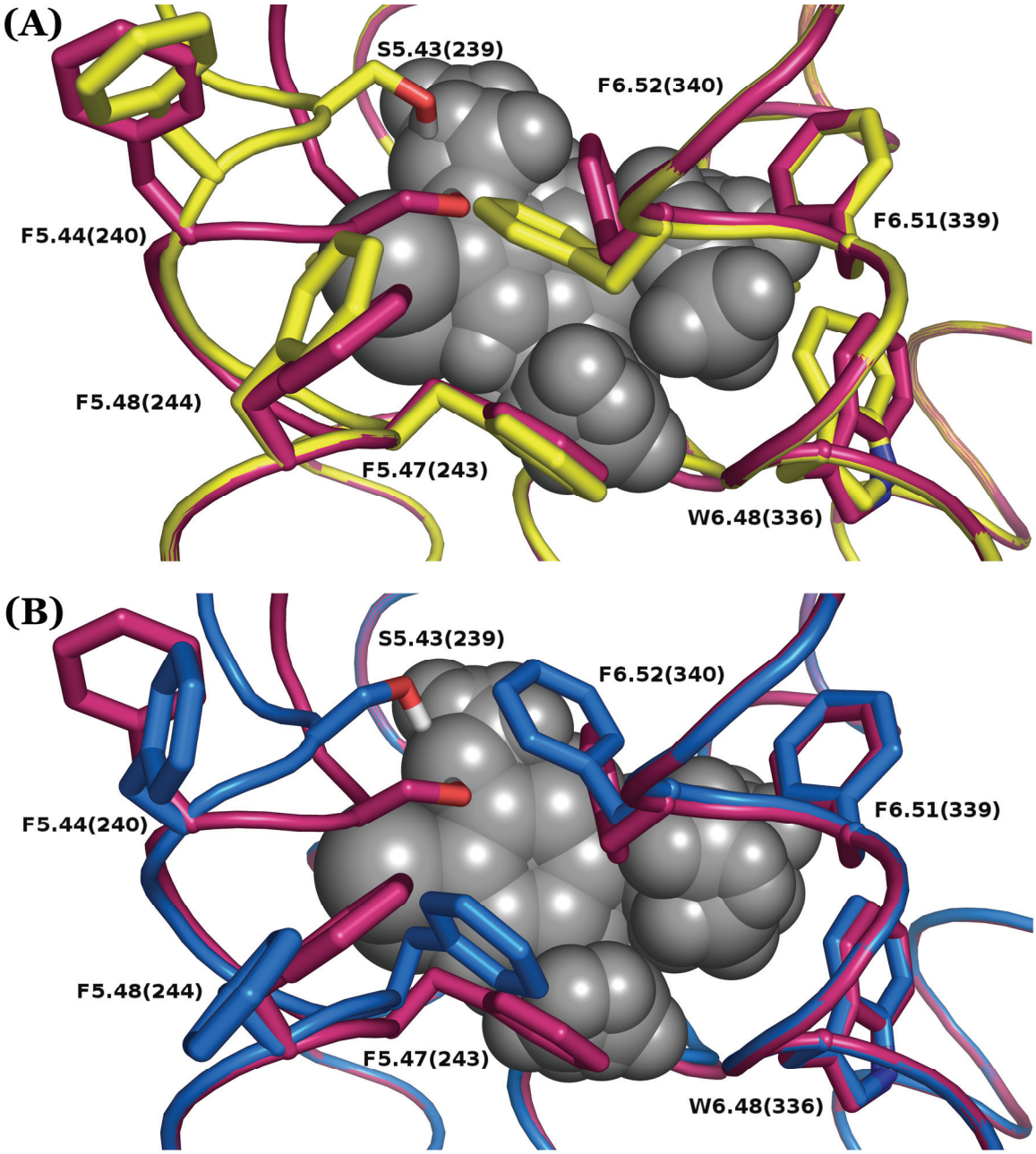


Figure 4.2 Differential receptor accommodation by PEAs and PIA enantiomers. **(A)**, (*R*)-DOB (red) and 2C-B (yellow); **(B)**, (*R*)-DOB (red) and (*S*)-DOB (blue). The cartoon for the associated receptor complement backbone for each ligand is similarly colored. The ligands in each panel are superimposed as grey space-filling spheres. The extracellular side of the receptor is toward the top of the figure, TM5 of the receptor is in the left foreground, and TM6 of the receptor in right foreground.

Differential receptor accommodation observed with virtual docking simulations of representative PEA/PIA and (*R/S*)-PIA pairs

By comparing the virtual docking simulations of a representative pair of PEA and PIA compounds, namely 2C-B vs. (*R*)-DOB, we observed marked differences in the conformations of receptor residues in TM5 and TM6 of the h5-HT_{2A} receptor homology model. As Figure 4.2A illustrates, the root mean square (RMS) displacements of F6.52(340), F5.44(240), S5.43(239), and a portion of the backbone of TM5 not in the force field aggregate set (and thus allowed to move within the simulations) are all approximately 2-3Å. The aromatic residues F5.48(244) and W6.48(336) showed slight changes in conformation (about 1Å), whereas F5.47(243) and F6.51(339) showed very little differences in conformation (<0.5Å) following docking of the achiral 2C-B.

The comparison of (*R*)- vs. (*S*)-DOB shown in Fig. 4.2B illustrates similar marked differences in the conformations of TM5 and TM6 receptor residues, particularly for F6.52(340), S5.43(239), and the non-aggregate portion of TM5, as well as F5.44(240), F5.47(243), and F5.48(244), all with RMS displacements of 2-3Å. The conformations of W6.48(336) and F6.51(339) within these two docked ensembles are again virtually superimposable (RMSD < 0.5Å).

4.1.2. Discussion

With the consideration that high affinity and functional activity at 5-HT_{2A} is necessary for hallucinogenic action, these data appear to support the limited anecdotal reports of the psychopharmacology of the phenylalkylamines in that the (*R*)-isomer of a PIA is generally more potent than the (*S*)-isomer and that the PIAs are more potent than their PEA homologues (Shulgin and Shulgin, 1991). Although there may be pharmacokinetic factors involved in the anecdotally observed differences in PIA and PEA potencies, these data indicate that the robustness of the receptor-mediated second messenger signal, namely intrinsic activity of PI hydrolysis, may be a factor in these differences, an effect first observed with 4-trifluoromethyl phenylalkylamine analogues (Nichols *et al.*, 1994). These data are consistent with previous studies showing no difference in binding affinity for PEAs and their corresponding racemic PIAs (Johnson *et*

al., 1987; Glennon *et al.*, 1992; Nash *et al.*, 1994), although these studies found no difference in intrinsic activities between PEA/PIA pairs.

The comparison of PIA enantiomer pairs, which should not share similar pharmacokinetics, also displayed similar changes in intrinsic activities in cells expressing human 5-HT_{2A} receptors (A20 cells; 150 fmol/mg), although binding affinity and functional potencies for the (*S*)-isomers at both receptors were also lower in all cases. The lack of statistically discernable differences at the rat 5-HT_{2A} receptors (GF6 cells; 5500 fmol/mg) are likely due to the high receptor expression and thus large receptor reserve for coupling to the PLC messaging system (Kenakin, 1997; Kurrasch-Orbaugh *et al.*, 2003b). A more thorough evaluation of these pharmacological data and their implications for the second messenger signaling of 5-HT_{2A} receptor agonists can be found in the thesis of Jason Parrish (Parrish, 2006).

Of more relevance to the evaluations here, the accommodation of the receptor binding site to these different ligands was investigated utilizing virtual docking and subsequent constrained energy minimization simulations performed with representative compounds. One must be careful in linking robust activity in the PI hydrolysis assay system to the general activity of hallucinogens in humans (Parrish, 2006; Parrish and Nichols, 2006). With that caveat, these data indicate that, at least for phenylalkylamines, the ability to stabilize an active state of the receptor and generate a robust signal through the PLC pathway is correlated with activity in humans. Therefore, a biophysical understanding of possible differences in how these ligands interact with the 5-HT_{2A} receptor and affect its conformation may help to explain this correlation.

As discussed in the introduction, the solvent-accessible putative binding site of the 5-HT_{2A} receptor involves contacts primarily with transmembrane domains TM3, TM5, and TM6 (van Rhee and Jacobsen, 1996; Roth *et al.*, 1998; Kristiansen, 2004). Furthermore, G-protein coupling and subsequent activation of the PLC second messenger system is dependent on G-protein association with residues in the third intracellular loop (IL3), the cytoplasmic domain that connects TM5 and TM6 (Kubo *et al.*, 1988; Oksenberg *et al.*, 1995; Hill-Eubanks *et al.*, 1996). As the putative binding site resides in the extracellular side of the transmembrane domain of the receptor, and if the

energetically favorable secondary structure is to be maintained, it is possible that slight movements of the extracellular side of the helices may translate into large movements on the intracellular side. Therefore, ligand-receptor interactions that affect the orientation or conformation of receptor residues on TM5 or TM6 may transduce down and affect the structure of IL3 and coupling to the heterotrimeric G-protein complex.

The different receptor accommodations for representative pairings of PEA/PIAs or (*R/S*)-PIAs show major perturbations in the conformations of receptor residues in TM5 and TM6 believed to be involved in ligand binding and activity. S5.43(239) is believed to be important for the binding and activity of ring-substituted tryptamines (Johnson *et al.*, 1997; Shapiro *et al.*, 2000) and implicated by previous docking simulations to be interacting with the 5-oxygen of phenylalkylamines (Chambers and Nichols, 2002). An entire section of the TM5 backbone including S5.43(239) shows a dramatic change in orientation, particularly in the comparison of 2C-B and (*R*)-DOB (Figure 4.2A). The disruption of the α-helical structure of TM5 is likely due to the artificial constraint set between S5.43(239) and the 5-oxygen, as well as the aggregate that keeps most of the protein from moving but is involved in energy calculations. These artificial constraints and aggregates are necessary due to the limitations of current modeling software to find and properly weight hydrogen bonds. All docking and energy simulations utilized the same initial receptor structure, constraints, and aggregates.

In addition to an altered accommodation of this polar contact on TM5, the packing of several aromatic residues of TM5 and TM6 was affected as well. Previous studies have indicated that the orientation of this cluster of residues, particularly F5.47(243), W6.48(336), and F6.52(340), may be essential for the transduction of the agonist-stabilized active state towards G-protein coupling/activation, although mutations of F5.44(240) F5.48(244), and F6.51(339) were not found to affect phenylalkylamine agonist binding or activity (Choudhary *et al.*, 1995; Roth *et al.*, 1997b; Shapiro *et al.*, 2000). In the comparisons of 2C-B to (*R*)-DOB and of (*R/S*)-DOB, we observed dramatic shifts in the orientations of F6.52(340) (Figure 4.2A). We also observed a perturbation of F5.47(243) in comparing 2C-B to (*R*)-DOB, although much less so in the comparison of (*R*)- and (*S*)-DOB (Figure 4.2B).

Thus, residues that are believed to be involved in binding the ligand or stabilizing the receptor active state(s), S5.43(239), F5.47(243), and F6.52(340) show major perturbations when the (*S*)-enantiomer or the PEA binds, as compared to the (*R*)-PIA. By contrast, F6.51(339) shows virtually no perturbation with virtual binding of any of the ligands, consistent with mutagenesis studies indicating that F6.51(339) does not directly interact with small molecule agonists (Choudhary *et al.*, 1995; Roth *et al.*, 1997b; Shapiro *et al.*, 2000). These results from virtual docking simulations appear to follow the trends of previous mutagenesis studies of 5-HT_{2A} receptors and affirm the utility of our *in vitro*-activated h5-HT_{2A} homology model for providing qualitative support of pharmacological data.

4.2. Aromatic and hydrogen bond interactions of novel and classic ligands with residues in TM6 of the 5-HT_{2A} receptor

The goal of this specific aim was to begin to provide empirical evidence for the ligand-receptor interactions observed in virtual docking simulations to our h5-HT_{2A} homology model. These investigations also provided an opportunity to characterize a new class of super-potent 5-HT_{2A} receptor agonists, namely *N*-benzyl analogues of phenylalkylamines ("N-Benzyls"). This library of compounds was further utilized to explore the SAR of these compounds and the receptor binding site.

Virtual docking simulations of the N-Benzyls to the h5-HT_{2A} receptor displayed interactions expected of the phenethylamine pharmacophore. A novel interaction between a TM6 residue previously identified to interact only with antagonists in the 5-HT_{2A} receptor and the *N*-aryl moiety of the N-Benzyls was observed. This aromatic interaction was hypothesized to be key to the high affinity and potency of these compounds. Furthermore, most N-Benzyls appeared to benefit from having a polar substitution in the 2-position of the new aryl moiety; these compounds showed increased affinity and potency at 5-HT_{2A} receptors. Some docking orientations placed these 2-position substituents near a TM6 polar residue that had previously been predicted, but not verified, to interact with the carbonyl group of ergolines (*e.g.* LSD). This hydrogen bond interaction was hypothesized to be contributing to the high affinities and potencies

of the N-Benzyls and ergolines. Site-directed mutagenesis of these TM6 aromatic and polar residues was therefore performed to investigate these hypotheses. Some of this work was a collaborative project with Jason Parrish and it should be noted that most of the binding and functional data at rat 5-HT_{2A} receptors were acquired by Dr. Parrish. The results of studies of the mutations of F6.51(339) and F6.52(340) have been published in Molecular Pharmacology (Braden *et al.*, 2006) and portions of the results and discussion sections of that manuscript are used here by permission. Compounds used for this specific aim are displayed in Figure 4.3.

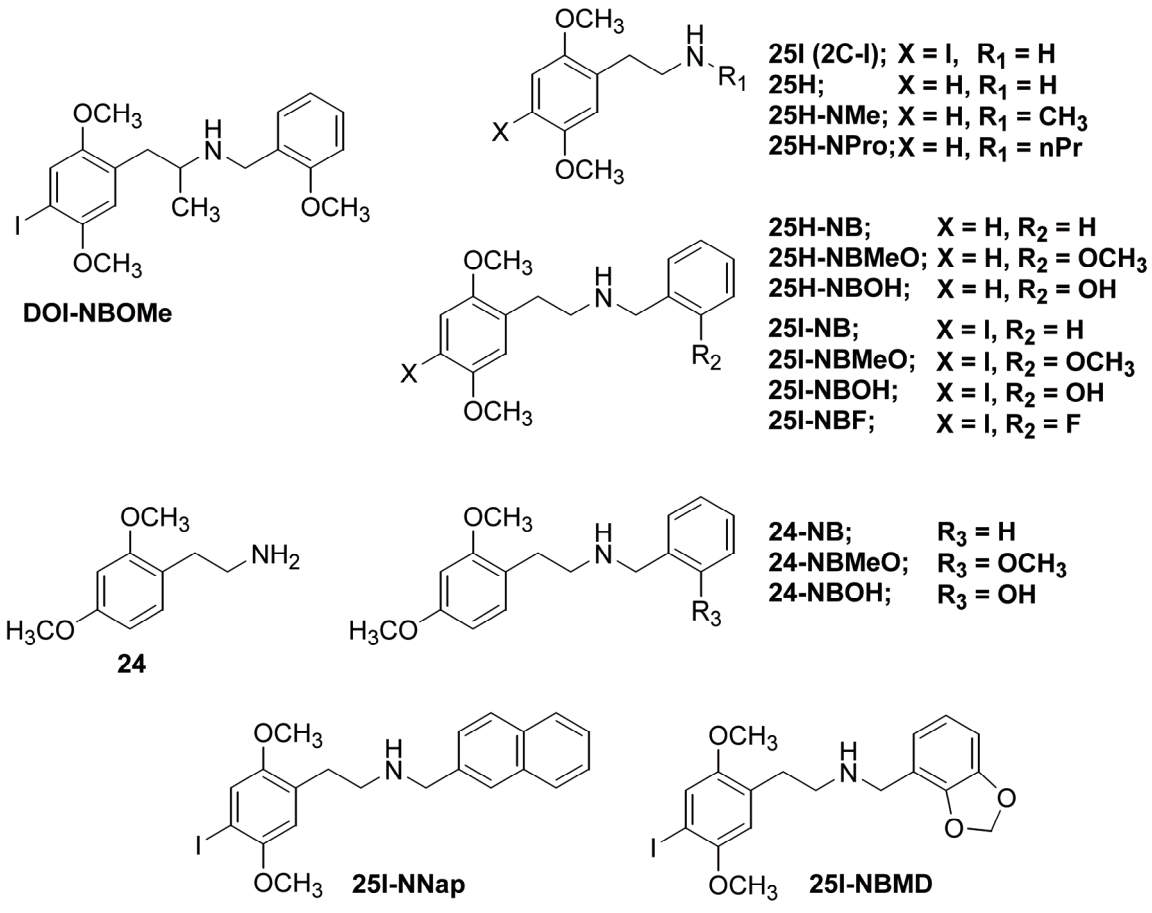


Figure 4.3 Structures of phenylalkylamines and N-Benzyls used in Specific Aim 2

4.2.1. Results

Aromatic *N*-substitution on phenylalkylamines is sufficient and necessary for dramatic increases in affinity at the rat 5-HT_{2A} receptor

As Table 4.3 indicates, increasing steric bulk on the amine of 25H with a methyl or *n*-propyl group to yield 25H-NMe and 25H-NPr was detrimental to the binding affinity at the rat 5-HT_{2A} receptor. In stark contrast, the addition of an *N*-benzyl group to 2CH to yield 2CH-NB produced a 13-fold increase in affinity, whereas the addition of an *N*-(2-methoxy)benzyl or *N*-(2-hydroxy)benzyl group to yield 25H-NBOMe and 25H-NBOH produced 190- and 82-fold increases in affinity, respectively. With the exceptions of the *N*-(2-methoxy)benzyl analogue of DOI (DOI-NBOMe), and the *N*-(2-napthyl)methyl analogue of 25I (25I-NNap) all *N*-arylmethyl analogues of phenylalkylamines followed this trend of increased binding affinity at the rat 5-HT_{2A} receptor. Although not indicated in Table 4.3, the N-Benzyls were also very selective (>1000-fold) for 5-HT_{2A} receptors over 5-HT_{1A} and moderately selective (up to 35-fold) for 5-HT_{2A} over 5-HT_{2C} (see Appendix).

***N*-Benzyl analogues of phenethylamines are potent agonists at the r5-HT_{2A} receptor**

Several of the test ligands were examined at cloned r5-HT_{2A} receptors for their ability to stimulate the hydrolysis of radiolabeled phosphatidylinositides (PI), as also shown in Table 4.3. All compounds tested were relatively potent agonists at the cloned rat 5-HT_{2A} receptor and possessed robust intrinsic activities, with the exception of DOI-NBOMe, 25I-NB 25I-NNap, and 25I-NBF, which were weak partial agonists. Dramatic increases of functional potency at the PI hydrolysis assay system were observed with the *N*-benzyl analogues of the phenethylamines 25H and 24 (about 50- to 100-fold vs. the parent compound). Although analogues of the phenethylamine 25I did not have dramatic increases in potency (*ca*. 2- to 8-fold), 25I-NOMe has the highest affinity and functional potency of any 5-HT_{2A} receptor agonist reported to date.

Table 4.3 Effect of *N*-alkyl or *N*-aryl phenylalkylamine substitution on binding and functional activity at the rat 5-HT_{2A} receptor. Data are presented as the mean and (SEM) from nonlinear regression fits of a single binding site model for K_i values and normalized variable slope (typically 0.6-1.4) sigmoidal dosage-response curves for estimates of EC50 and intrinsic activity. All data are from at least three independent experiments. A typical experiment would show 10-20 fold stimulation by 5-HT over basal for PI hydrolysis assays.

Drug	K_i	r5-HT_{2A}R PI Hydrolysis	
	r5-HT_{2A} (nM) (±)-[^{125}I]DOI	EC50 (nM)	Intrinsic Activity (% 5-HT)
25H	227 (39)	12877 (1930)	82(8)
25H-NMe	1286 (64)	ND	ND
25H-NPr	734 (30)	ND	ND
25H-NB	17.5 (1.9)	ND	ND
25H-NBOMe	1.19 (0.17)	81.2 (3.8)	81 (0.4)
25H-NBOH	2.76 (0.40)	141 (21)	66 (2)
24	202 (19)	4034 (260)	67 (8)
24-NB	28.5 (2.9)	ND	ND
24-NBOMe	0.68 (0.12)	51.0 (6.7)	72(1)
24-NBOH	0.67 (0.01)	74.0 (6.7)	82(4)
DOI	0.58 (0.06)	19.2 (2.6)	77 (3)
DOI-NBOMe	1.08 (0.21)	36.1 (2.7)	43 (3)
25I	0.62 (0.08)	19.0 (2.6)	59 (4)
25I-NB	0.31 (0.03)	12.0 (0.7)	37 (2)
25I-NNap	3.74 (0.52)	> 1 μM	25 @ 10 μM
25I-NBOMe	0.087 (0.010)	2.50 (0.55)	78 (6)
25I-NBOH	0.12 (0.02)	6.34 (0.18)	71 (2)
25I-NBF	0.28 (0.04)	23.2 (1.2)	32 (3)
25I-NBMD	0.19 (0.02)	8.2 (1.6)	68 (7)

ND not determined

Virtual docking of *N*-Benzyls and ergolines to the h5-HT_{2A} receptor homology model reveals possible aromatic and hydrogen bond contacts with TM6

As the examples in Figure 4.4 illustrate, unconstrained virtual docking simulations and subsequent constrained molecular dynamics and energy minimization simulations produced docking orientations that position the ergolines and the classic phenethylamine pharmacophore of N-Benzyls in a manner to indicate interactions with previously predicted and/or observed TM6 residues. These include the aromatic interaction with F6.52(340), as observed above in Specific Aim 1, as well as the hydrogen bond interaction of N6.55(343) with the carbonyl oxygen of ergolines (Figure 4.4B), as observed in the work of Jim Chambers (Chambers and Nichols, 2002). A novel π-π interaction was further observed between the *N*-arylmethyl moiety of the N-Benzyls and F6.51(339) (Figure 4.4A), a residue previously implicated to be involved only in the binding of antagonists to the 5-HT_{2A} receptor (Roth *et al.*, 1997b). Centroid distance measurements between the phenethylamine pharmacophore and F6.52(340) ranged from 4.6 to 4.7Å, whereas those between the *N*-benzyl moiety and F6.51(339) ranged between 4.8 to 4.9Å. Although not clearly evident in Figure 4.4A, these orientations further place the only polar residue in this TM region, N6.55(343), in a position to suggest it may be available for a hydrogen bond with the polar 2-position substituent of the *N*-aryl moiety of the N-Benzyls.

The F6.51(339)L and F6.52(340)L mutations within the h5-HT_{2A} receptor both strongly affect the binding affinities of the *N*-benzyl analogues, whereas F6.52(340)L strongly affects only the smaller classic ligands

K_D values for [^{125}I]DOI at wild type and F6.51(339)L mutant 5-HT_{2A} receptors were 0.78 ± 0.01 nM and 0.86 ± 0.14 nM, respectively. K_D values for [^{3}H]ketanserin at wild type, F6.51(339)L, and F6.52(340)L mutant 5-HT_{2A} receptors were 1.10 ± 0.12 nM, 11.1 ± 3.6 nM, and 0.40 ± 0.02 nM, respectively. As anticipated from the work of Roth and colleagues (1997), we were also not able to determine a K_D value for [^{125}I]DOI at the h5-HT_{2A}/F340L receptor. Furthermore, due to the high (11 nM) K_D for [^{3}H]ketanserin at h5-HT_{2A}/F339L, it was not practical to use this radioligand for assays at this receptor.

Figure 4.4 Illustrative cross-eyed stereopair representations of ligand poses from virtual docking experiments in the h5-HT_{2A} receptor with **(A)**, 25I-NBOH, showing proposed π-π interactions between the N-benzyl moiety and F339, and the aryl portion of the phenethylamine and F340, and **(B)**, *d*-LSD, showing an aromatic interaction between the indole region of LSD and F340, and no aromatic interaction with F339. Ligands are shown as space-filling spheres, and receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM6 in the foreground, TM5 on the left, TM3 in the right background, and the extracellular face of the receptor toward the top of the figure. TMs 1, 2, 4, and 7 are not displayed.

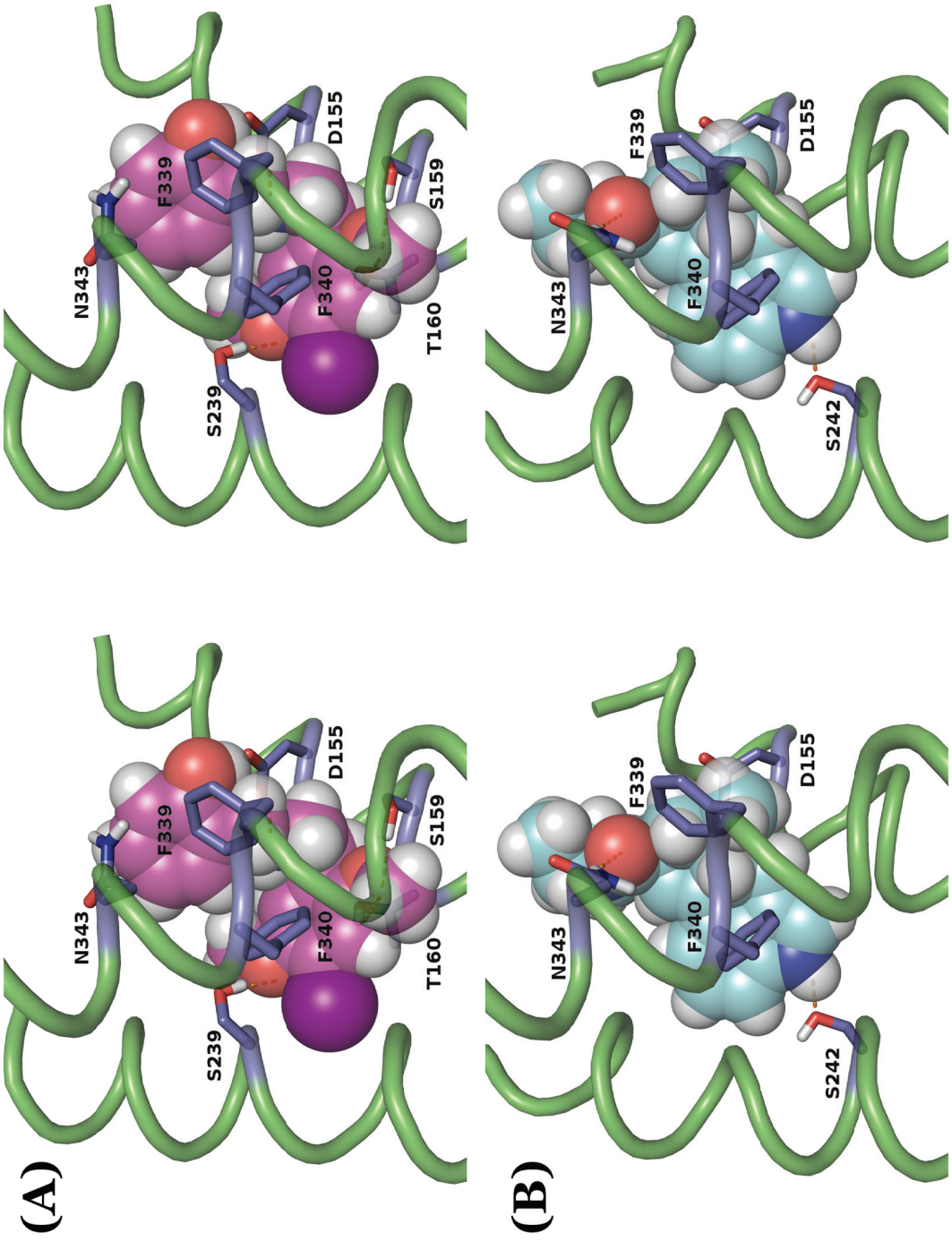

(A)
N343
F339
D155
S159
F340
T160
S239
(B)
N343
F339
D155
F340
S242

Figure 4.4

Therefore, competition binding assays were performed with [^{125}I]DOI at h5-HT$_{2A}$/F339L receptors, [^{3}H]ketanserin at h5-HT$_{2A}$/F340L receptors, and both radioligands at wild type receptors. In order to control for differences in wild type K_i values from different radioligand displacement, mutant receptor K_i values were compared only to wild type receptor values utilizing the same radioligand.

As Table 4.4 and Figure 4.5A show, the F6.51(339)L mutation had relatively weak (<10-fold) detrimental effects on the affinities of all the classic and smaller ligands except for the phenethylamine 25H and the 5-substituted tryptamines 5-HT and 5-MeO-DMT. With the exception of LSD and mescaline, all compounds tested had binding affinities that were statistically distinguishable from wild type ($p < 0.01$ from two-tailed Student's T-tests of pK_i values). The mutation had weak detrimental effects on binding when a hydrophobic *N*-alkyl substitution on the amine of 25H was present (about 5- to 6-fold), but when an *N*-arylmethyl group was attached, as seen with 25H-NB, 25H-NBOMe, and 25H-NBOH, there was a dramatic about 40- to 700-fold decrease in affinity compared to the wild type. A similar marked detrimental effect was observed between 24 and 24-NB, 24-NBOMe, and 24-NBOH, with a nearly 100-fold decrease in affinity for the latter three *N*-benzyl analogues. The *N*-benzyl analogues of 25I were not as strongly affected, with 25I-NNap, 25I-NBOMe, 25I-NBOH, and 25I-NBF showing 30- to 50-fold decreases in binding affinities, and 25I-NB and 25I-NMD showing only about 10-fold decreases in affinity.

As Table 4.5 and Figure 4.5B show, the F6.52(340)L mutation had comparatively weak (<10-fold) detrimental effects only on the phenethylamines mescaline, 25H, 24, and 25I, in addition to the aliphatic analogues 25H-NMe and 25H-NPro, and the aromatic analogue 25I-NBMD. Most of the other *N*-arylmethyl derivatives had about 100-fold decreases in binding affinities. The strongest detrimental effects were seen with 5-HT (>1000-fold), psilocin (160-fold), and 5-MeO-DMT (500-fold). The aromatic analogues followed a trend similar to that seen with the phenethylamines in the F6.51(339)L mutation. All compounds tested had binding affinities that were statistically distinguishable from the wild type ($p < 0.01$ from two-tailed Student's T-tests of pK_i values).

Table 4.4 Effect of the F6.51(339)L mutation on binding to the h5-HT_{2A} receptor. K_i values are presented as the mean and (SEM) in nM from at least three independent experiments. Except where indicated (†), all unpaired two-tailed Student T-tests generated $p<0.01$ for differences in pK_i between mutant and wild type receptors tested with the same radioligand.

Drug	(±)-[^{125}I]DOI		
	h5-HT_{2A} K_i (nM)	**h5-HT_{2A}/F339L K_i (nM)**	**ΔΔG° (kcal/mol)**
5-HT	4.84 (0.2)	59.6 (10.0)	1.5
***d*-LSD**	0.40 (0.02)	0.60 (0.12)†	0.2
psilocin	11.8 (1.2)	28.6 (4.3)	0.5
5-MeO-DMT	7.54 (1.06)	129 (15)	1.7
mescaline	1500 (200)	4500 (600)†	0.6
25H	377 (67)	5800 (730)	1.6
25H-NMe	1900 (250)	8720 (670)	0.9
25H-NPr	1300 (150)	7860 (770)	1.1
25H-NB	68.1 (10.6)	2722 (470)	2.2
25H-NBOMe	2.83 (0.31)	1435 (192)	3.5
25H-NBOH	3.73 (0.45)	2642 (455)	3.9
24	300 (29)	1013 (190)	0.7
24-NB	26.6 (2.7)	1768 (339)	2.5
24-NBOMe	1.71 (0.34)	252 (49)	3.0
24-NBOH	1.51 (0.20)	310 (57)	3.1
25I	0.73 (0.06)	2.63 (0.32)	0.8
25I-NB	0.25 (0.05)	3.1 (0.1)	1.3
25I-NNap	4.83 (0.55)	160 (31)	2.1
25I-NBOMe	0.044 (0.006)	2.08 (0.35)	2.3
25I-NBOH	0.061 (0.012)	1.84 (0.16)	2.0
25I-NBF	0.26 (0.05)	15.2 (1.7)	2.4
25I-NBMD	0.049 (0.008)	0.29 (0.03)	1.1

† $p>0.01$

Table 4.5 Effect of the F6.52(340)L mutation on binding to the h5-HT_{2A} receptor. K_i values are presented as the mean and (SEM) in nM from at least three independent experiments. All unpaired two-tailed Student T-tests generated $p<0.01$ for differences in pK_i between mutant and wild type receptors tested with the same radioligand.

Drug	[^{3}H]Ketanserin		
	h5-HT_{2A} K_i (nM)	**h5-HT_{2A}/F340L K_i (nM)**	**ΔΔG° (kcal/mol)**
5-HT	77.6 (13.8)	193000 (36300)	4.6
***d*-LSD**	0.81 (0.16)	13.01 (1.09)	1.6
psilocin	22.8 (4.0)	3659 (243)	3.0
5-MeO-DMT	49.2 (3.2)	23726 (4726)	3.7
mescaline	14640 (2447)	62425 (10485)	0.9
25H	2000 (310)	16000 (3160)	1.2
25H-NMe	5930 (92)	43920 (2270)	1.2
25H-NPr	3600 (640)	9815 (940)	0.6
25H-NB	184 (33)	6698 (1031)	2.1
25H-NBOMe	11.0 (0.5)	689 (107)	2.5
25H-NBOH	11.6 (1.7)	277 (40)	1.9
24	1000 (180)	8390 (1200)	1.3
24-NB	71.9 (3.0)	3320 (360)	2.3
24-NBOMe	5.24 (1.01)	700 (14)	2.9
24-NBOH	2.83 (0.36)	290 (14)	2.7
25I	4.52 (0.30)	28.9 (4.8)	1.1
25I-NB	0.28 (0.02)	27.0 (1.8)	2.7
25I-NNap	6.68 (1.02)	270 (268)	2.1
25I-NBOMe	0.15 (0.03)	4.3 (0.76)	2.1
25I-NBOH	0.068 (0.012)	1.58 (0.17)	1.9
25I-NBF	0.19 (0.03)	37.9 (1.3)	3.1
25I-NBMD	0.21 (0.03)	0.94 (0.17)	0.9

Figure 4.5 Effects on binding affinities of the **(A)** F6.51(339)L, and **(B)** F6.52(340)L mutations in the h5-HT_{2A} receptor. These bar graphs display the $\Delta\Delta G°$ values (kcal/mol) derived from the data of Tables 4.4 and 4.5 (see Methods). Larger negative (upward) values in these graphs indicate a greater negative effect of the mutation on binding affinity. The dashed line at -1.0 indicates an arbitrary threshold for "weak" effects. ** indicates $p<0.01$ for values of ΔpKi from unpaired two-tailed Student T-tests.

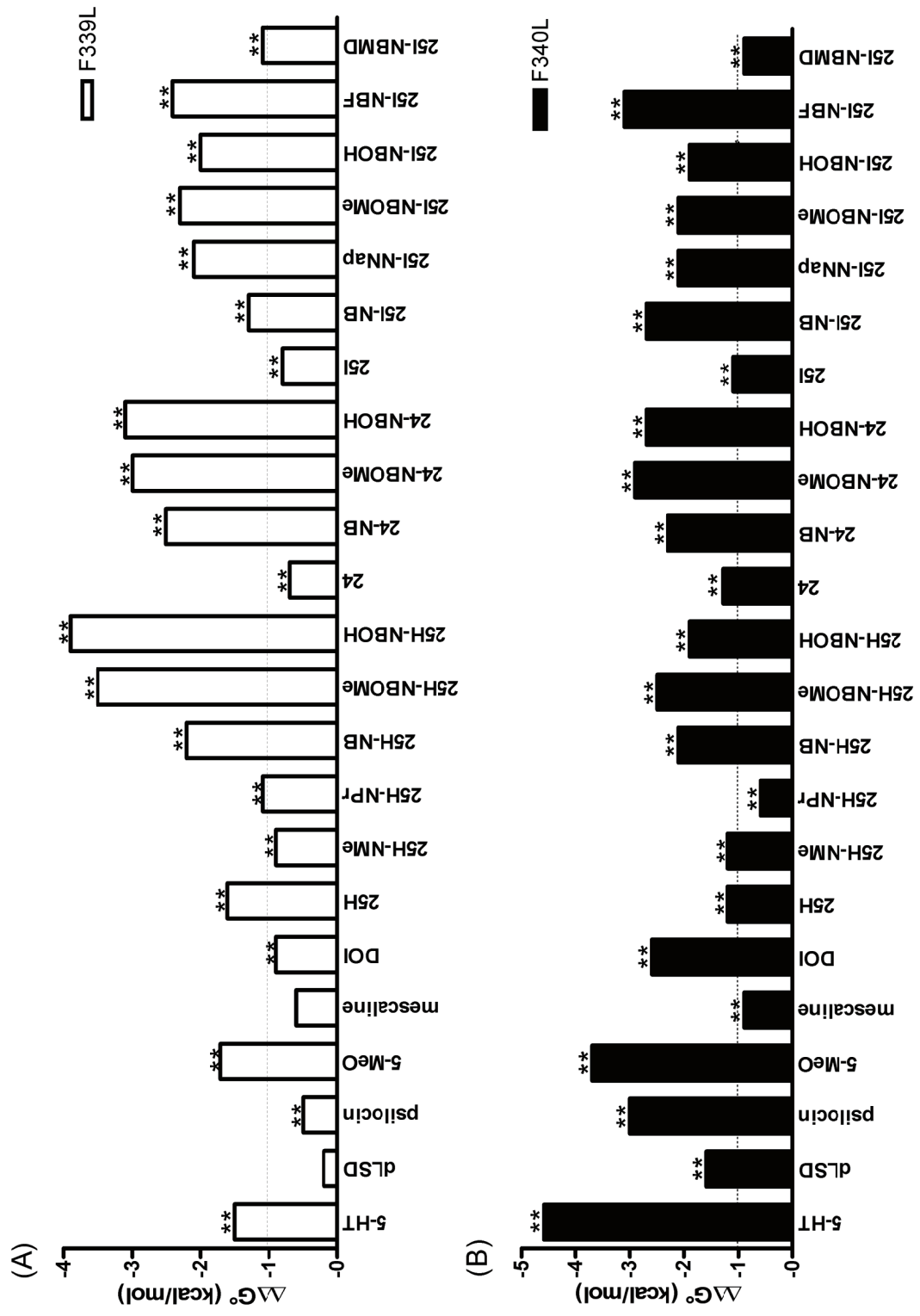

(A)
F339L
ΔΔG° (kcal/mol)
0
-1
-2
-3
-4
5-HT
dLSD
psilocin
5-MeO
mescaline
DOI
25H
25H-NMe
25H-NPr
25H-NB
25H-NBOMe
25H-NBOH
24
24-NB
24-NBOMe
24-NBOH
25I
25I-NB
25I-NNap
25I-NBOMe
25I-NBOH
25I-NBF
25I-NBMD
(B)
F340L
ΔΔG° (kcal/mol)
0
-1
-2
-3
-4
-5
5-HT
dLSD
psilocin
5-MeO
mescaline
DOI
25H
25H-NMe
25H-NPr
25H-NB
25H-NBOMe
25H-NBOH
24
24-NB
24-NBOMe
24-NBOH
25I
25I-NB
25I-NNap
25I-NBOMe
25I-NBOH
25I-NBF
25I-NBMD

Figure 4.5

Both the F6.51(339)L and F6.52(340)L mutations within the h5-HT_{2A} receptor adversely affect the ability of test compounds to stimulate PI hydrolysis to different degrees

As Table 4.6 and Figure 4.6 indicate, the F6.51(339)L mutation had relatively weak detrimental effects ("weak" here is defined as <10-fold) on the potency of LSD and the unsubstituted phenethylamine agonists mescaline, 25H, 24, and 25I. The tryptamines 5-HT, psilocin, and 5-MeO-DMT were moderately affected. This mutation produced strong detrimental effects (about 100-fold or greater), however, with all of the *N*-benzyl phenethylamine analogues. Some detrimental effects on intrinsic activity of the smaller agonists were observed with this mutation, but generally were weak ("weak" here is defined as ΔInt.Act. <25%). As evident in the EC50 values, stronger detrimental effects on intrinsic activity were observed with all of the *N*-benzyl analogues of phenethylamines. For some of the *N*-benzyl analogues, this change is so dramatic that they are transformed from nearly full agonists at the wild type receptor into very weak partial agonists, possibly antagonists, by the mutation.

The F6.52(340)L mutation had marked detrimental effects on the potency of all compounds tested, particularly the tryptamines 5-HT, psilocin, and 5-MeO-DMT. Due to the extreme loss of potency for 5-HT at this mutant receptor and solubility limitations of 5-HT, the normalization concentration of serotonin may not be maximally stimulating and thus all intrinsic activity values indicated at this mutant receptor may be slightly elevated, and all negative ΔInt.Act. values may therefore also be underestimated. With this caveat, the F6.52(340)L mutation had strong detrimental effects on intrinsic activity (efficacy) of all the smaller classic agonists. Interestingly, the *N*-benzyl analogues of 25H and one analogue of 24 also showed relatively strong (about 100-fold or greater) decreases in intrinsic activity, whereas none of the 25I analogues showed any strong decreases. As with the F6.51(339)L mutation, the compounds most dramatically affected by the F6.52(340)L mutation were transformed from nearly full agonists at the wild type receptor to very weak partial agonists in the mutant receptor.

Table 4.6 Effects of the F6.51(339)L and F6.52(340)L mutations on h5-HT_{2A} receptor-mediated PI hydrolysis. Data are presented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity values from at least three independent experiments. A typical experiment would show 4-10 fold stimulation by 5-HT over basal. Except where indicated (†), all two-way ANOVA tests with Bonferroni post-tests generated $p<0.01$ for values of pEC50 and Intrinsic Activity between mutant and wild type receptors.

Table 4.6

Drug	h5-HT_{2A}		h5-HT_{2A}/F339L		h5-HT_{2A}/F340L	
	EC50 PI Hydrolysis (nM)	**Intrinsic Activity (% 5-HT)**	**EC50 PI Hydrolysis (nM)**	**Intrinsic Activity (% 5-HT)**	**EC50 PI Hydrolysis (nM)**	**Intrinsic Activity (% 5-HT)**
5-HT	5.17 (0.97)	100	92.4 (10.5)	100	9840 (460)	100
***d*-LSD**	0.22 (0.04)	84 (3)	1.36 (0.23)	55 (5)	15.7 (2.9)	20 (5)
psilocin	7.29 (0.72)	105 (9)	130 (18)	44 (8)	4530 (810)	9 (1)
5-MeO-DMT	4.33 (0.78)	98 (4)	416 (71)	74 (5)	5255 (970)	15 (4)
mescaline	1120 (220)	83 (5)	11330 (990)	82 (7)†	78800 (3870)	30 (1)
25H	1020 (14)	96 (10)	10350 (1650)	78 (1)†	141000 (39540)	12 (4)
25H-NBOMe	15.3 (3.7)	88 (6)	3410 (390)	27 (4)	1340 (53)	43 (5)
25H-NBOH	23.5 (1.8)	100 (6)	11270 (760)	32 (6)	2160 (500)	28 (3)
24	830 (200)	83 (5)	4080 (580)	66 (4)	109300 (37700)	17 (1)
24-NBOMe	4.00 (0.80)	89 (6)	1440 (280)	55 (5)	2030 (200)	66 (8)†
24-NBOH	5.42 (0.66)	84 (4)	5620 (29)	49 (8)	700 (140)	31 (3)
25I	2.54 (0.18)	82 (3)	22.8 (2.7)	72 (5)†	99.5 (5.3)	38 (2)
25I-NB	1.96 (0.12)	66 (2)	1090 (350)	14 (2)	260 (40)	82 (1)†
25I-NBOMe	0.44 (0.07)	81 (4)	28.0 (5.2)	51 (4)	26.8 (4.2)	84 (7)†
25I-NBOH	0.19 (0.03)	86 (5)	42.3 (6.5)	45 (6)	14.6 (2.9)	82 (7)†
25I-NBF	1.55 (0.21)	87 (11)	150 (25)	8 (1)	410 (33)	81 (6)†
25I-NBMD	1.07 (0.20)	72 (3)	91.0 (30.9)	11 (1)	145 (25)	70 (5)†

† $p > 0.01$

Figure 4.6 Effects on EC50 and intrinsic activity of PI hydrolysis functional activity by F339(6.51)L (open bars) and F340(6.52)L (solid bars) mutations in the h5-HT_{2A} receptor. These bar graphs display the ΔpEC50 and ΔInt. Act. values derived from the data of Table 4.6 (see Methods). All intrinsic activity values are normalized to serotonin stimulation and thus it is not possible to obtain ΔInt.Act. values for 5-HT. Positive ΔInt.Act. values for 25I-NB and 25I-NBOMe at the h5-HT2A/F340L receptor were not significantly different from WT and are not shown for formatting reasons. Dashed lines at -1.0 and -25% indicate an arbitrary threshold for "weak" effects. ** indicates $p<0.01$ for values of ΔpEC50 and ΔInt.Act. from two-way ANOVA tests with Bonferroni post-tests.

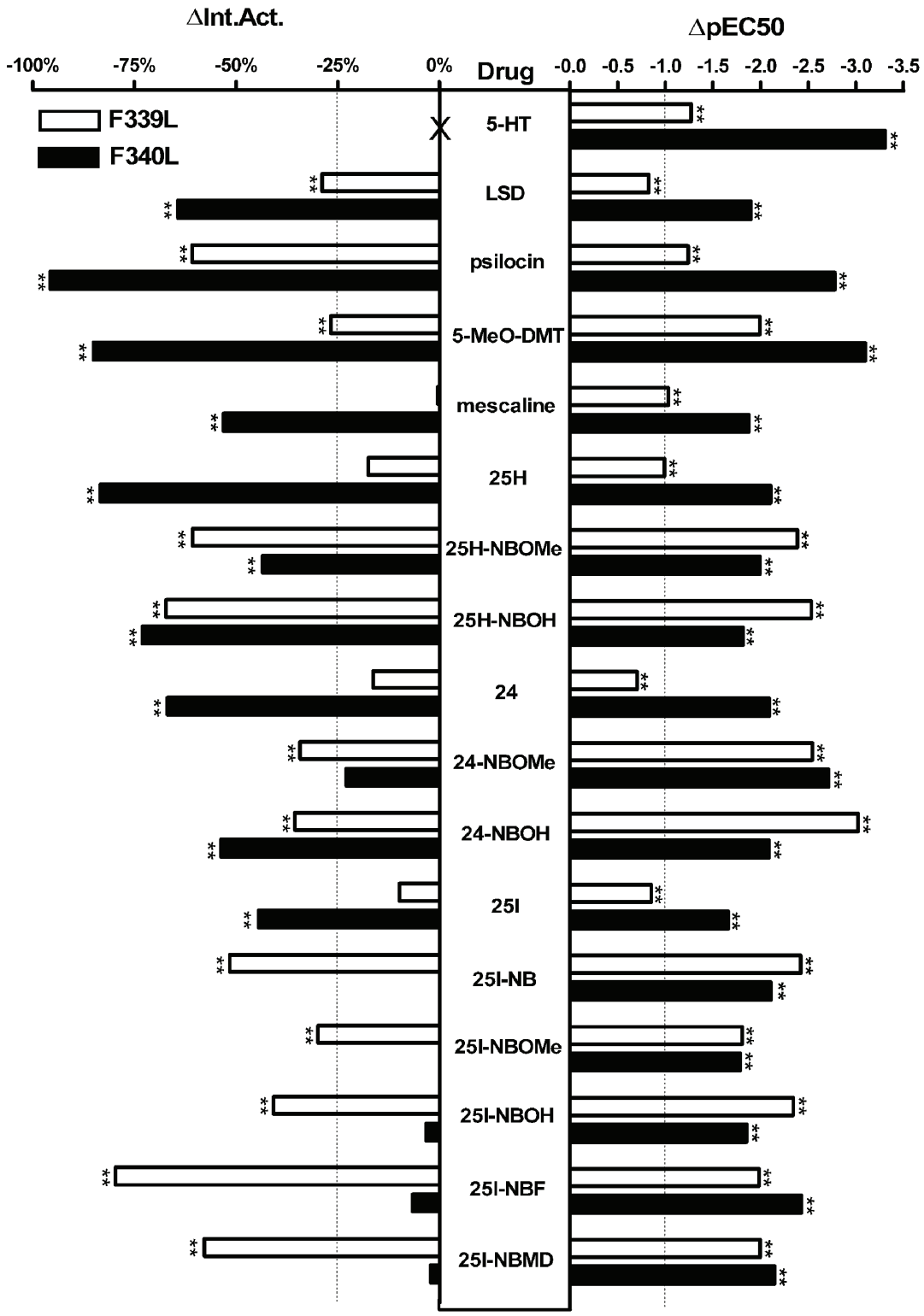

ΔInt.Act.
ΔpEC50
-100%
-75%
-50%
-25%
0%
Drug
-0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
F339L
F340L
5-HT
LSD
psilocin
5-MeO-DMT
mescaline
25H
25H-NBOMe
25H-NBOH
24
24-NBOMe
24-NBOH
25I
25I-NB
25I-NBOMe
25I-NBOH
25I-NBF
25I-NBMD

Figure 4.6

The N6.55(343)A mutation within the h5-HT_{2A} receptor does not affect the affinity or potency of ergolines and N-Benzyls

The contribution of a hydrogen bond to the standard free energy of binding (ΔG°) has been determined to be -0.5 to -2 kcal/mol (Fersht, 1988). As Table 4.7 and Figure 4.8 indicate, the N6.55(343)A mutation did not affect the binding of ergolines to a degree indicating the loss of a hydrogen bond. The binding affinity of LSD actually increased two-fold whereas those of the piperidide, methylisopropyl, and *trans*-dimethylazetidide analogues LSDPip, Lamide and *S,S*-trans were not statistically distinguishable from wild type. The N-Benzyl compounds 24-NBOH and 25I-NBF have polar substituents at the benzyl 2-position and had a decrease in binding affinity at the h5-HT_{2A}/N343A mutant receptor commensurate with the loss of a hydrogen bond. However, the unsubstituted 25I-NB was similarly affected and none of the other N-Benzyls tested with a polar 2-position substituent showed changes in affinity that were statistically discernable from wild type. Moreover, the compounds most affected by the N6.55(343)A mutation appear to be a limited selection of smaller, classic 5-HT_{2A} receptor agonists, namely serotonin, DOM, and mescaline and its ethyl and isopropyl analogues, escaline and isoproscaline.

As Table 4.8 and Figure 4.8 show, the N6.55(343)A mutation had relatively weak effects (about 5-fold or less) on functional potency of PI hydrolysis by the ergolines and N-Benzyls, although the intrinsic activity of LSD was reduced by 28%. 25I-NB had a 15% increase in intrinsic activity. The most dramatic change in functional potency at PI hydrolysis, of greater than 100-fold, was observed with the endogenous ligand serotonin. There was an approximately 10-fold reduction in potency for the tryptamines 5-MeO and psilocin. Although the functional potency of DMT and DET was not reduced, all tryptamines tested had a 20-40% loss in intrinsic activity. Functional potency was decreased 10- to 20-fold, and intrinsic activity was reduced 20-30% for mescaline, escaline, and isoproscaline; none of the other phenylalkylamines, however, were affected. Structures for the additional compounds used for these mutant receptors are indicated in Figure 4.7.

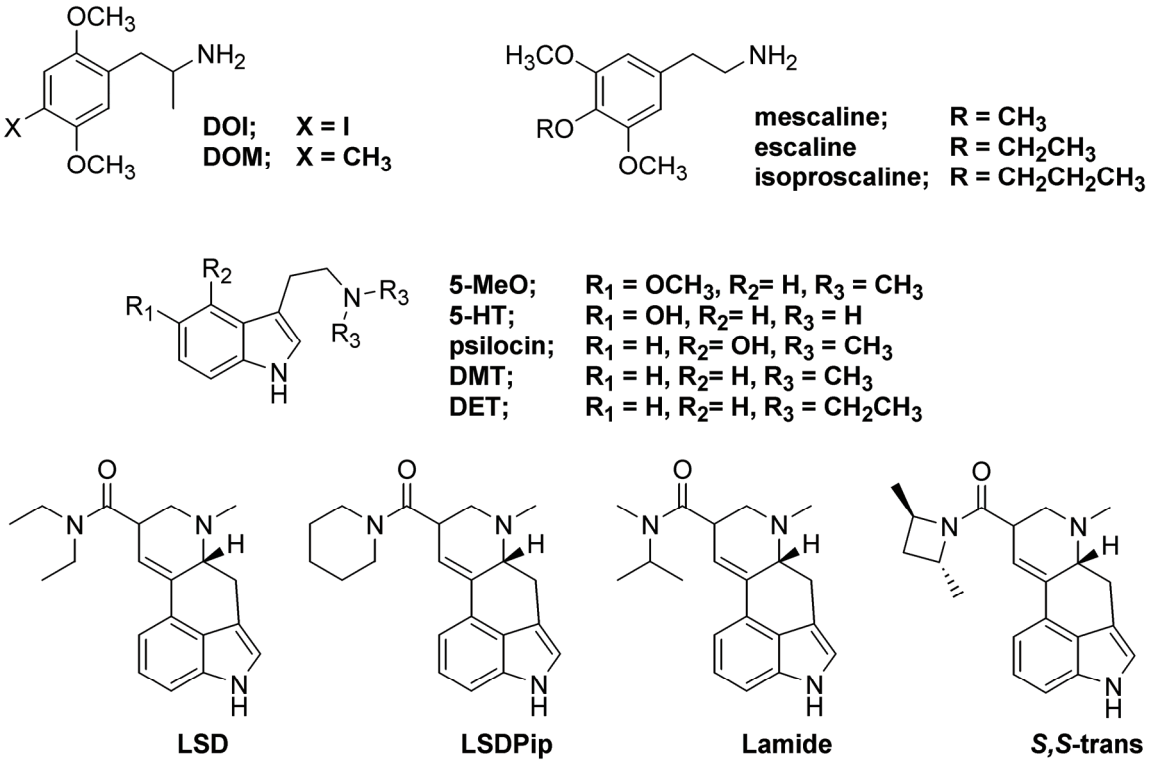


Figure 4.7 Structures for additional compounds used in Specific Aim 2.

Table 4.7 Effect of the N6.55(343)A mutation on binding to the h5-HT_{2A} receptor. K_i values are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments. * indicates unpaired two-tailed Student T-tests that generated $p<0.05$ for differences of pK_i between mutant and wild type receptors.

Drug	(±)-[^{125}I]DOI		
	h5-HT_{2A} K_i (nM)	**h5-HT_{2A}/N343A K_i (nM)**	**-ΔΔG° (kcal/mol)**
LSD	0.40 (0.02)	0.20 (0.03)*	0.4
LSDPip	0.32 (0.02)	0.47 (0.09)	-0.2
Lamide	0.33(0.07)	0.36 (0.06)	0.0
***S,S*-trans**	0.54 (0.11)	0.36 (0.04)	0.2
5-MeO	7.5 (1.1)	6.9 (0.9)	0.1
5-HT	4.8 (0.2)	61.3 (7.0)*	-1.5
psilocin	11.8 (1.2)	15.8 (1.5)	-0.2
DMT	75.1 (6.0)	54.0 (6.5)	0.2
DET	64.9 (1.3)	51.4 (8.7)	0.1
DOI	0.64 (0.06)	0.72 (0.08)	-0.1
DOM	5.9 (1.0)	25.1 (3.6)*	-0.9
mescaline	1500 (250)	7360 (510)*	-0.9
escaline	520 (100)	960 (180)	-0.4
isoproscaline	470 (66)	1860 (330)*	-0.8
24-NBOMe	1.7 (0.3)	3.0 (0.6)	-0.3
24-NBOH	1.5 (0.2)	3.7 (0.7)*	-0.5
25I-NBOMe	0.044 (0.006)	0.067 (0.010)	-0.2
25I-NBOH	0.061 (0.012)	0.049 (0.005)	0.1
25I-NBF	0.26 (0.05)	0.64 (0.13)*	-0.5
25I-NB	0.25 (0.05)	0.63 (0.12)*	-0.6

* $p < 0.05$

Table 4.8 Effect of the N6.55(343)A mutation on h5-HT_{2A} receptor-mediated PI hydrolysis. Data are presented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity values from at least three independent experiments. A typical experiment would show 4-10 fold stimulation by 5-HT over basal. Except where indicated (†), all unpaired two-tailed Student T-tests generated $p<0.01$ for differences of pEC50 between mutant and wild type receptors, whereas * indicates where these tests generated $p<0.01$ for Intrinsic Activity.

Drugs	h5-HT_{2A}		h5-HT_{2A}/N343A	
	EC50 PI Hydrolysis (nM)	**Intrinsic Activity (% 5-HT)**	**EC50 PI Hydrolysis (nM)**	**Intrinsic Activity (% 5-HT)**
LSD	0.22 (0.04)	84 (3)	0.38 (0.05)	57 (3)*
LSDPip	0.75 (0.04)	79 (7)	3.1 (0.3)	63 (9)
Lamide	0.46 (0.02)	79 (6)	2.0 (0.2)	60 (10)
***S,S*-trans**	0.32 (0.09)	85 (9)	0.45 (0.07)†	64 (5)
5-MeO	4.3 (0.8)	98 (4)	62.0 (7.1)	81 (2)*
5-HT	5.2 (1.0)	100	586 (47)	100
psilocin	7.3 (0.7)	105 (9)	90.6 (12.7)	82 (2)
DMT	188.1 (6.2)	71 (1)	357 (64)†	31 (7)*
DET	69.1 (1.1)	73 (6)	127 (16)	38 (1)*
DOI	0.69 (0.09)	84 (2)	2.2 (0.4)	91 (1)*
DOM	6.4 (1.7)	86 (2)	28.6 (0.8)	93 (3)
mescaline	1117 (223)	83 (5)	13633 (712)	49 (14)*
escaline	275 (54)	100 (3)	4340 (250)	75 (4)*
isoproscaline	220 (34)	101 (3)	3660 (572)	78 (3)*
24-NBOMe	4.0 (0.8)	89 (6)	13.3 (2.0)	79 (4)
24-NBOH	5.4 (0.7)	84 (4)	27.5 (5.5)	85 (4)
25I-NBOMe	0.4 (0.1)	81 (4)	0.66 (0.13)†	80 (3)
25I-NBOH	0.19 (0.03)	86 (5)	0.59 (0.10)	87 (5)
25I-NBF	1.6 (0.2)	79 (9)	9.1 (1.5)	69 (5)
25I-NB	2.0 (0.1)	66 (2)	9.1 (1.4)	80 (4)*

† EC50 $p > 0.05$; * Int.Act. $p < 0.05$

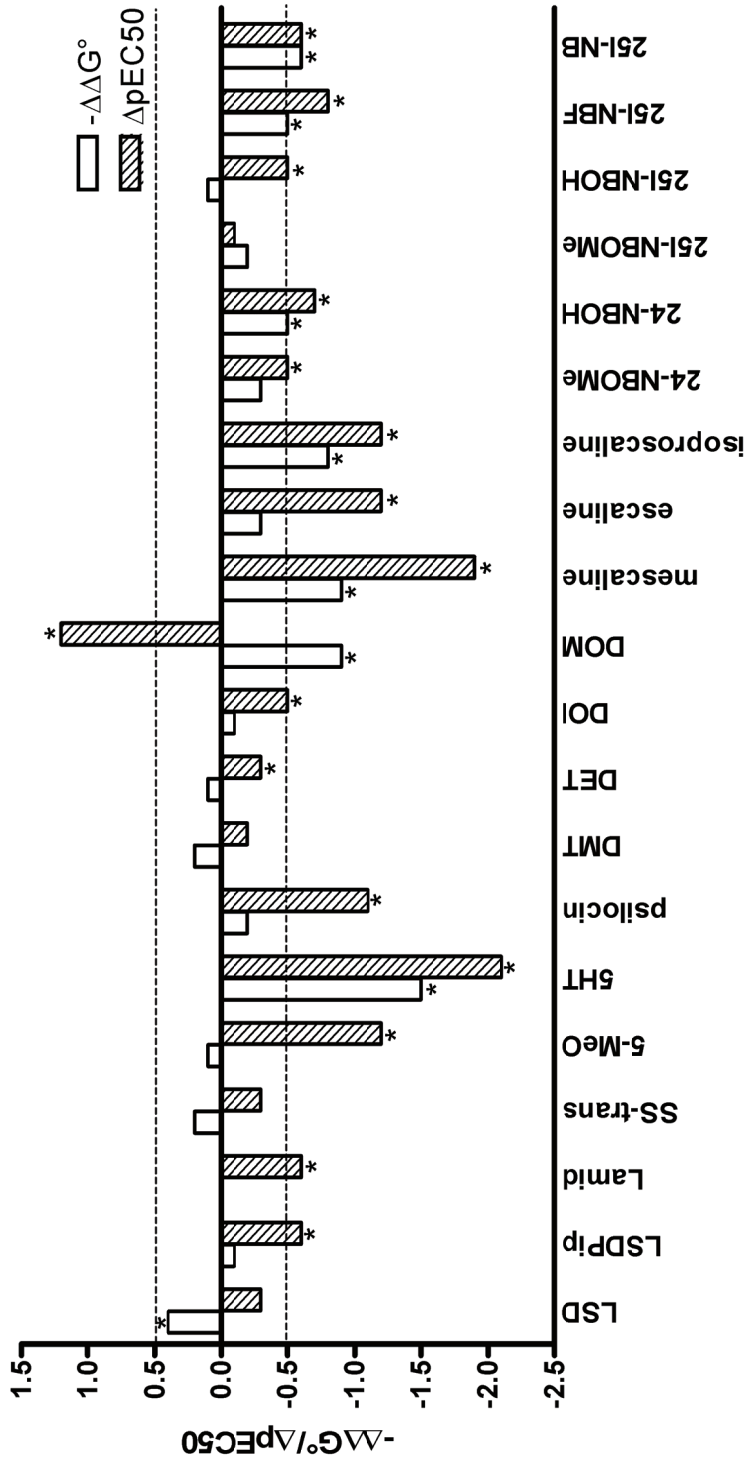


Figure 4.8 Effects of the N6.55(343)A mutation on binding affinity and functional potency at the h5-HT_{2A} receptor. Data are shown as changes in the standard Gibbs free energy of binding (kcal/mol; open bars) and changes in functional potency for PI hydrolysis (relative units; shaded bars) from the data of Tables 4.7 and 4.8. Dashed lines at ±0.5 indicate minimum threshold for the energetics of a hydrogen bond (Fersht, 1988) or an arbitrary threshold for "weak" effects on functional potency. * indicates where two-tailed Student's T-tests generated $p<0.05$.

4.2.1. Discussion

While working on Specific Aim 1, it became apparent that the artificial constraints employed and the resulting interactions we observed in virtual docking simulations were not always empirically sound. Thus, the utility of the model shifted from merely qualitative support for pharmacological data to a hypothesis generator for validation and optimization of the topology of the model, and to investigate further the predicted ligand-receptor interactions. By empirically supporting the fidelity of our h5-HT_{2A} receptor homology model binding site, and justifying the use of certain constraints, we could be confident of its utility for exploring the subtle aspects of ligand binding and stabilization of receptor active states, as well as directing ligand synthesis.

The first opportunity to validate the topology of our h5-HT_{2A} receptor homology model came with the characterization of a new class of super potent analogues of phenylalkylamines. Initially, we wished to determine whether the enhanced effects of *N*-(2-methoxy)benzyl substitution on phenethylamines previously reported (Pertz *et al.*, 1999; Elz *et al.*, 2002) would be observed in our heterologous expression systems. We further sought to verify whether the aromatic character of this substitution was necessary by comparing two *N*-alkyl substituted phenethylamines. A previous study on the effects of substitution on the alkylamine of phenylalkylamines and indoleethylamines on binding to the rat 5-HT_{2A} receptor found that alkyl substitution was generally not tolerated (Glennon *et al.*, 1994). The same study also found that *N*-arylmethyl analogues of the phenethylamine 2C-B showed 2- to 3-fold increased affinity; however, the underlying basis for this effect was not evaluated, and none of their ligands were tested for functional activity.

As Tables 4.3, 4.4, and 4.6 show, the *N*-arylmethyl analogues of phenethylamines ("N-Benzyls") are potent and efficacious agonists with high affinity for both rat and human 5-HT_{2A} receptors. These analogues also depend on the aromatic character of the *N*-substitution for the often dramatic improvements of affinity and potency observed compared to the unsubstituted phenethylamines. The *N*-methyl and *N*-(*n)*-propyl analogues of 25H had decreased affinity at all receptors tested, as well as decreased potencies at the human 5-HT_{2A} receptor. Although *N*-arylmethyl analogues of 25I did

not show as marked an increase in affinity or potency as those of 24 or 25H, they still possessed extremely high affinities at the h5-HT_{2A} receptor, with affinities as low as 40 pM. Moreover, the presence of a polar group on the 2-position of the *N*-arylmethyl group also appeared to improve affinity and potency (*e.g.* 25I-NB vs. 25I-NBOM). We recognized, therefore, that the aromatic ring of the *N*-benzyl moiety was enhancing activity through some specific biophysical property, which we believe to be a π-π interaction with an aromatic residue in the receptor. Furthermore, we predicted that a hydrogen bond also may be occurring between the polar *N*-benzyl 2-position oxygen and a polar residue in the receptor to enhance activity.

Previous virtual dockings of phenethylamines and related classic agonists have produced low-energy ensemble structures with orientations placing F6.52(340) in a position to interact with the aromatic ring of the ligand through a π-π interaction, as discussed in Specific Aim 1. Those findings were consistent with previous mutagenesis results indicating the cognate residue in other homologous receptors is solvent-accessible (Javitch *et al.*, 1998) and is involved in an essential π-π interaction with agonists (Choudhary *et al.*, 1993; Choudhary *et al.*, 1995; Cho *et al.*, 1995; Huang *et al.*, 1995; Roth *et al.*, 1997b). Other mutagenesis studies, however, indicate that this residue is not solvent accessible (Chen *et al.*, 1999), does not interact with agonists, and/or may be interacting with some antagonists (Choudhary *et al.*, 1993; Choudhary *et al.*, 1995; Cho *et al.*, 1995; Wieland *et al.*, 1999), or is not involved in receptor activation (Ward *et al.*, 1999). Docking orientations produced in this study again placed the aromatic ring of the phenethylamine pharmacophore in a position to interact with F6.52(340), as well as positioning the polar methoxy groups in ways that indicated interactions with other residues identified by site-directed mutagenesis to be involved in 5-HT_{2A} agonist binding (Wang *et al.*, 1993; Kristiansen *et al.*, 2000; Shapiro *et al.*, 2000) and as illustrated in Figure 4.2.

A potential π-π interaction also was identified between F6.51(339) and the novel *N*-benzyl moiety of the ligand. Mutation of the cognate residue in a variety of other GPCRs has indicated that residue 6.51 is: (1) located near retinal in bovine rhodopsin (Nakayama and Khorana, 1991); (2) solvent accessible (Javitch *et al.*, 1998; Chen *et al.*,

1999); and (3) may be essential for a π-π interaction with both agonists and antagonists (Nardone and Hogan, 1994; Cho *et al.*, 1995; Huang *et al.*, 1995; Ward *et al.*, 1999; Chen *et al.*, 1999). With respect specifically to the 5-HT_{2A} receptor, previous data seemed to indicate that this residue was not within the agonist-binding pocket and was not involved in the binding of agonists or partial agonists (Choudhary *et al.*, 1993), but rather stabilized binding of the antagonist ketanserin (Choudhary *et al.*, 1995; Roth *et al.*, 1997b).

In addition to these aromatic interactions of TM6 residues of the 5-HT_{2A} receptor with the N-Benzyls, some docking orientations placed the polar 2-oxygen of these ligands in proximity to N6.55(343), the only TM residue in this region with a polar side chain. A similar interaction had previously been observed between N6.55(343) and the carbonyl oxygen of ergolines, although this hydrogen bond was thought to mediate antagonist action at 5-HT_{2A} receptors (Chambers and Nichols, 2002). Mutation of the cognate asparagine residue in human adenosine A_{2a} receptors decreased the binding of both agonists and antagonists (Kim *et al.*, 1995).

In order to test the hypotheses that both F6.51(339) and F6.52(340) might be contributing to a π-π interaction with N-Benzyls and that N6.55(343) may be contributing a hydrogen bond with N-Benzyls and ergolines, separate mutations of these three residues were performed in the h5-HT_{2A} receptor. A semi-conservative phenylalanine to leucine mutation was chosen to eliminate aromaticity, while conserving steric bulk and hydrophobicity of the residues to reduce possible global effects of the mutations (Fersht *et al.*, 1987). Similarly, a non-disruptive asparagine to alanine "deletion" mutation was chosen to eliminate the polar side chain group while maintaining some of the original side chain size. Concerns about potential global structural change were addressed by including several different chemical classes of classic 5-HT_2 agonists. Differential shifts in binding affinities between ligand classes should indicate more regional changes in the receptor structure rather than a global one affecting all ligand classes. Moreover, it has been shown that large differences in expression level can have marked effects on potency and intrinsic activity (Esbenshade *et al.*, 1995; Zhong *et al.*, 1996; Kenakin, 1997; Roth *et al.*, 1997b). Thus, the original high expression $Hh2A_{hi}$ cell line that had been

established with a [^{3}H]ketanserin B_{max} of about 9000 fmol/mg was used primarily for binding assays. HEK-293 cell lines stably expressing mutant receptors were chosen for more moderate expression, and a new cell line was developed with wild type expression comparable to the mutant h5-HT_{2A} receptor expressing cell lines. The newly constructed $Hh2A_{lo}$ cell population had a B_{max} of about 1600 fmol/mg, compared to expression levels of approximately 2200, 2500 and 1500 fmol/mg, respectively, for the mutant Hh2A/F339L, Hh2A/F340L, and Hh2A/N343A cell lines.

Differences in binding affinities between mutant and wild type receptors were assessed, as well as the functional repercussions of the F6.51(339)L and F6.52(340)L mutations in the PI hydrolysis second messenger system. The results of these experiments are illustrated in Figures 4.5 and 4.6, based on the data from Tables 4.4, 4.5 and 4.6. The trends observed in these figures seem to indicate that both F6.51(339) and F6.52(340) are interacting with the N-Benzyls, whereas F6.52(340) appears to be interacting mainly with *N*-unsubstituted phenethylamines and the other classic agonists. The $\Delta\Delta G°$ values of about 2-4 kcal/mol and centroid-centroid distances of about 4.6 to 4.9Å are consistent with the energy and orientation of a "T-shape" π-π interaction (Jorgensen and Severance, 1990). This trend is particularly evident in the weak effects of the F6.51(339)L mutation on EC50 and near absence of effects on intrinsic activity of the *N*-unsubstituted phenethylamines 25H, 24, and 25I. In stark contrast, the F6.51(339)L mutation had profound effects on both measures of function for the *N*-benzyl analogues of these phenethylamines, which were converted to very weak partial agonists at this mutant receptor.

The F6.52(340)L mutation affected the affinity and potency of nearly all the ligands tested, although to a different degree across different ligand classes. This mutant receptor still appears able to induce activation of PI hydrolysis in a dose-dependent manner, thus reducing concerns about massive global change of the receptor structure. The simple classic agonists also are turned into very weak partial agonists by this mutation. Surprisingly, the F6.52(340)L mutation does not appear to have an effect on intrinsic activities of the *N*-benzyl analogues of 25I, yet binding and potency are shifted

to a degree similar to the other *N*-benzyl analogues. Thus, an interaction with F6.51(339) in this series may be sufficient to produce a fully "active" receptor state.

If the hypothesis is valid that N6.55(343) interacts with the amide oxygen of ergolines and the polar ortho-substitution of the *N*-Benzyls, one would expect a marked decrease in binding affinity and/or functional activity at the h5-HT_{2A}/N343A mutant receptor for these compounds. Surprisingly, this effect was not observed. As indicated in Figure 4.8, the affinity for LSD actually increased slightly, the other ergolines and half of the N-Benzyls were indistinguishable from wild type, and the remaining N-Benzyls had only slight decreases; the potencies for the ergolines and most of the N-Benzyls were decreased, but only slightly. As with affinity, the changes of intrinsic activity were either slight or indistinguishable from wild type, with the exception of LSD, where there was a -25% shift, and 25I-NB, which actually increased slightly. Due to the 100-fold shift in functional potency of serotonin, the saturating dose of serotonin used for normalization purposes may not be fully stimulating. Although that was not generally observed in the raw counts for the assays of wild type and mutant receptor plates (data not shown), it may have artificially increased the calculated intrinsic activities of the other compounds at the h5-HT_{2A}/N343A mutant receptor.

Further confounding these results is the observation that a number of compounds that were not predicted from virtual docking simulations to interact directly with N6.55(343) do in fact have dramatic decreases in binding and/or activity. These smaller, more well known partial agonists are predicted to have binding orientations lower in the binding site, as seen with 2CB/DOB in Figure 4.2 of Specific Aim 1 and for the phenethylamine pharmacophore in Figure 4.4. Of particular interest are the ring-substituted tryptamines serotonin, psilocin, and 5-MeO-DMT, which have drastic (10- to 100-fold) changes in their potencies, whereas, with the exception of serotonin, their affinities and intrinsic activities are only slightly affected or indistinguishable from wild type. In contrast, the unsubstituted tryptamines DMT and DET have slight to indistinguishable changes in their affinities and potencies, yet there are moderately strong effects on their intrinsic activity. Moreover, a similarly confusing result is observed with

mescaline and its ethoxy and isopropoxy analogues. All three have a slight decrease in affinity and a moderate decrease in potency and intrinsic activity.

These results seem to indicate that an interaction of N6.55(343) with ergolines or N-Benzyls does not occur, or is not critical for binding and/or activity. There seems to be some indication that ring-substituted tryptamines may be able to adopt an orientation alternate to the conventional view in the agonist binding site so they can interact with N6.55(343). One could imagine the indole ring "flipping over" so that the indole nitrogen hydrogen would interact with the carbonyl oxygen of N6.55(343). Similarly, mescaline and its analogues may be adopting alternate or additional orientations that allow them to interact with this residue. Therefore, additional experiments with N(1)-substituted tryptamines at this mutant receptor may be necessary. Alternately, it is possible that N6.55(343) is an anchoring residue for the extracellular loop connecting TM4 and TM5 (EL2). The "capping" of the receptor by EL2 may alter ligand signaling or bring other receptor residues into contact with the ligand and could be altered by the N6.55(343)A mutant. This possibility is very difficult to model due to lack of accurate solvation of these residues in our work.

F6.51(339) and F6.52(340) are highly conserved and make up an evolutionarily constrained cascade of residues that mediate allosteric communication and receptor activation in GPCRs (Süel *et al.*, 2003), as well as being involved in a cluster of residues surrounding W6.48(336) that are believed to be a "toggle switch" for receptor activation (Shi *et al.*, 2002; Weinstein, 2005). Along with N6.55(343), these residues reside within transmembrane domain 6 (TM6), which is directly coupled to the third intracellular loop (IL3), between TM5 and TM6. As discussed in the introduction, this loop is implicated as important for the ability of the 5-HT_{2A} receptor (and all related GPCRs) to interact with and activate the appropriate G-proteins, and thus affect second messenger production, particularly $G\alpha_q$ activation of PI hydrolysis (Kubo *et al.*, 1988; Wess *et al.*, 1989; Oksenberg *et al.*, 1995; Hill-Eubanks *et al.*, 1996). Agonist or antagonist character, affinity, and potency of a particular ligand thus appears to be dependent on the ability of the ligand to interact with specific TM6 residues and induce or suppress movement of this helix. Although there is disagreement about the individual

contributions of F6.51 and F6.52 toward the binding and activity of agonists and antagonists across several amine-binding type A GPCRs, it appears from our data that within the h5-HT_{2A} receptor, involvement of either residue may be sufficient for agonist binding and receptor activation.

Moreover, although we have provided further evidence that F6.52(340) is likely not involved in the binding of the antagonist ketanserin (Roth *et al.*, 1997), our results do not preclude the possibility that this residue interacts with other classes of structurally diverse antagonists or partial agonists. We also have expanded and extended the findings of Pertz *et al.* (1999), and Elz *et al.* (2002), that *N*arylmethyl-phenethylamines, particularly those with a 2-methoxy or 2-hydroxy function on the benzyl moiety, represent a novel class of high affinity, potent, and modestly 5-HT_{2A}-selective receptor agonists.

Nevertheless, we believe our data are consistent with the general topology for the h5-HT_{2A} receptor reflected in our *in silico*-activated homology model of this receptor (Chambers and Nichols, 2002), particularly in the packing of aromatic residues around the ligand. Finally, and perhaps most important, we believe it may be possible to exploit the cognate residue at 6.51 in other GPCRs to design agonists with increased potency and intrinsic activity at not only 5-HT_{2A} receptors, but other GPCR neurotransmitter receptors as well.

4.3. Hydrogen bond interactions of tryptamines with polar residues in TM5 of the h5-HT_{2A} receptor

The primary goal of this specific aim was to explore further the topology of our h5-HT_{2A} receptor homology model. These investigations focused on providing empirical evidence for the orientations of tryptamines observed in virtual docking simulations. As with the dockings of phenylalkylamines observed in Specific Aims 1 and 2, the polar ring substitution of tryptamines was found to be oriented in a manner that would indicate the possibility of an interaction with a polar residue in TM5 of the h5-HT_{2A} receptor, namely Ser5.43(239). Additionally, the indole nitrogen was oriented in a manner to indicate an interaction with another polar TM5 residue, namely Ser5.46(242). Previous

investigations of interactions of tryptamines with Ser5.43(239) and Ser5.46(242) proposed conflicting roles for these residues (Kao *et al.*, 1992; Johnson *et al.*, 1993; Johnson *et al.*, 1994; Johnson *et al.*, 1997; Shapiro *et al.*, 2000). These residues are thus hypothesized to be specifically involved in the interactions with tryptamines as observed in our virtual docking simulations. Site-directed mutagenesis of Ser5.43(239) and Ser5.46(242), as well as reciprocal alteration of ligand structure, were utilized to investigate these hypotheses. Compounds used for this specific aim are displayed in Figure 4.9. below.

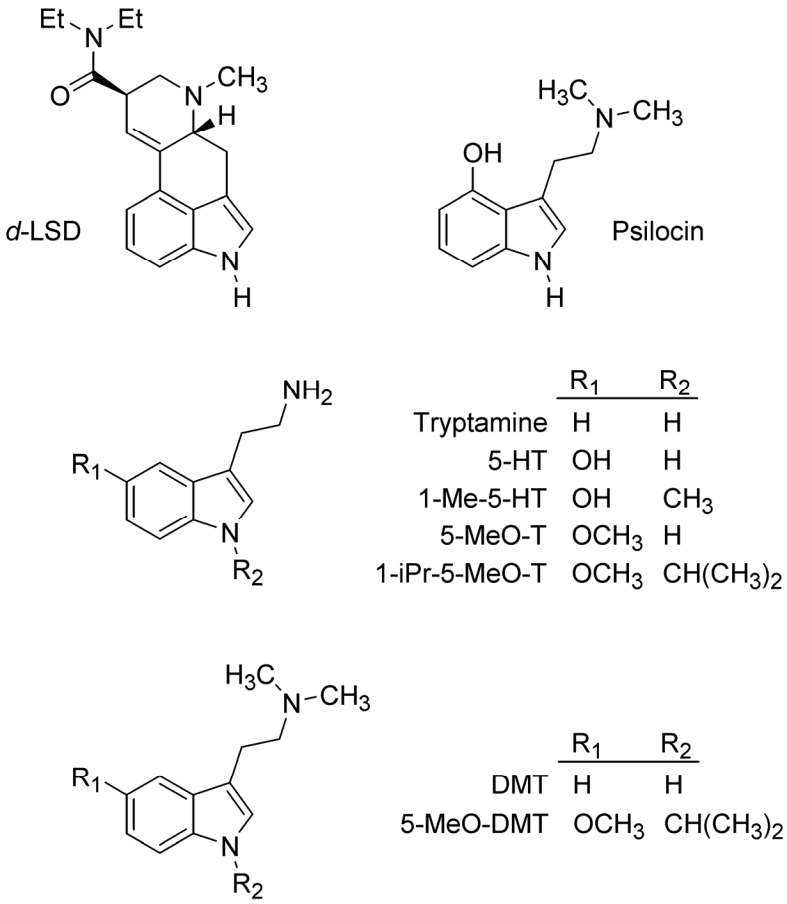


Figure 4.9 Tryptamine ligands used in Specific Aim 3

4.3.1. Results

Human S5.43(239)A and S5.46(242)A mutant 5-HT_{2A} receptors possess acceptable affinity and expression levels

Saturation isotherm binding assays with wild type h5-HT_{2A} receptors utilizing [^{3}H]ketanserin and [^{125}I]DOI yielded K_D values of 1.1 ± 0.12 nM and 0.78 ± 0.01 nM, respectively. S5.43(239)A mutant h5-HT_{2A} receptors yielded values of 2.18 ± 0.12 nM and 1.35 ± 0.08 nM, respectively, and S5.46(242)A mutant receptors yielded values of 0.71 ± 0.13 nM and 1.74 ± 0.17 nM, respectively. Receptor expressions based on B_{max} values from [^{3}H]ketanserin saturation binding assays were 8843 ± 858 fmol/mg for WT h5-HT_{2A} receptors, 3790 ± 557 fmol/mg for h5-HT_{2A}/S239A receptors, and 3215 ± 531 fmol/mg for h5-HT_{2A}/S242A receptors.

Virtual docking of tryptamines to an *in silico*-activated h5-HT_{2A} receptor homology model indicates that S5.43(239) may interact with polar substituents on the aromatic moiety of the ligand, whereas S5.46(242) may interact with the indole nitrogen

Figure 4.10 illustrates representative binding poses for virtual docking and subsequent energy minimization simulations of tryptamines in an *in silico*-activated homology model of the h5-HT_{2A} receptor. We observed final low energy binding poses for ligands that position their polar aromatic substituents in regions and orientations able to interact with h5-HT_{2A} receptor residues identified to be potentially important for agonist binding and activity (Chambers and Nichols, 2002). Of particular relevance for this study, the 4- or 5-oxygen atom of ring-substituted tryptamines is positioned to engage in hydrogen bonding with S5.43(239). Moreover, virtual docking simulations are consistent with an interaction between S5.46(242) and the indole nitrogen of the tryptamines, as suggested by others (Kao *et al.*, 1992; Johnson *et al.*, 1994). As Figure 4.10 further illustrates, LSD, an ergoline that shares structural features similar to the tryptamines, is also oriented to allow an interaction between the indole nitrogen and S5.46(242); however, there are no polar groups in LSD positioned to interact with S5.43(239).

Figure 4.10 Illustrative cross-eyed stereopair representation of ligand poses from virtual docking experiments with **(A)**, 5-MeO-DMT, and **(B)**, *d*-LSD, in the h5-HT_{2A} receptor showing predicted polar interactions between the ligand and receptor residues. Ligands are shown as space-filling spheres, whereas receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM5 on the left, TM6 in the foreground, TM3 in the right background, and with the extracellular face of the receptor toward the top of the figure. TMs 1, 2, 4, and 7 are not displayed.

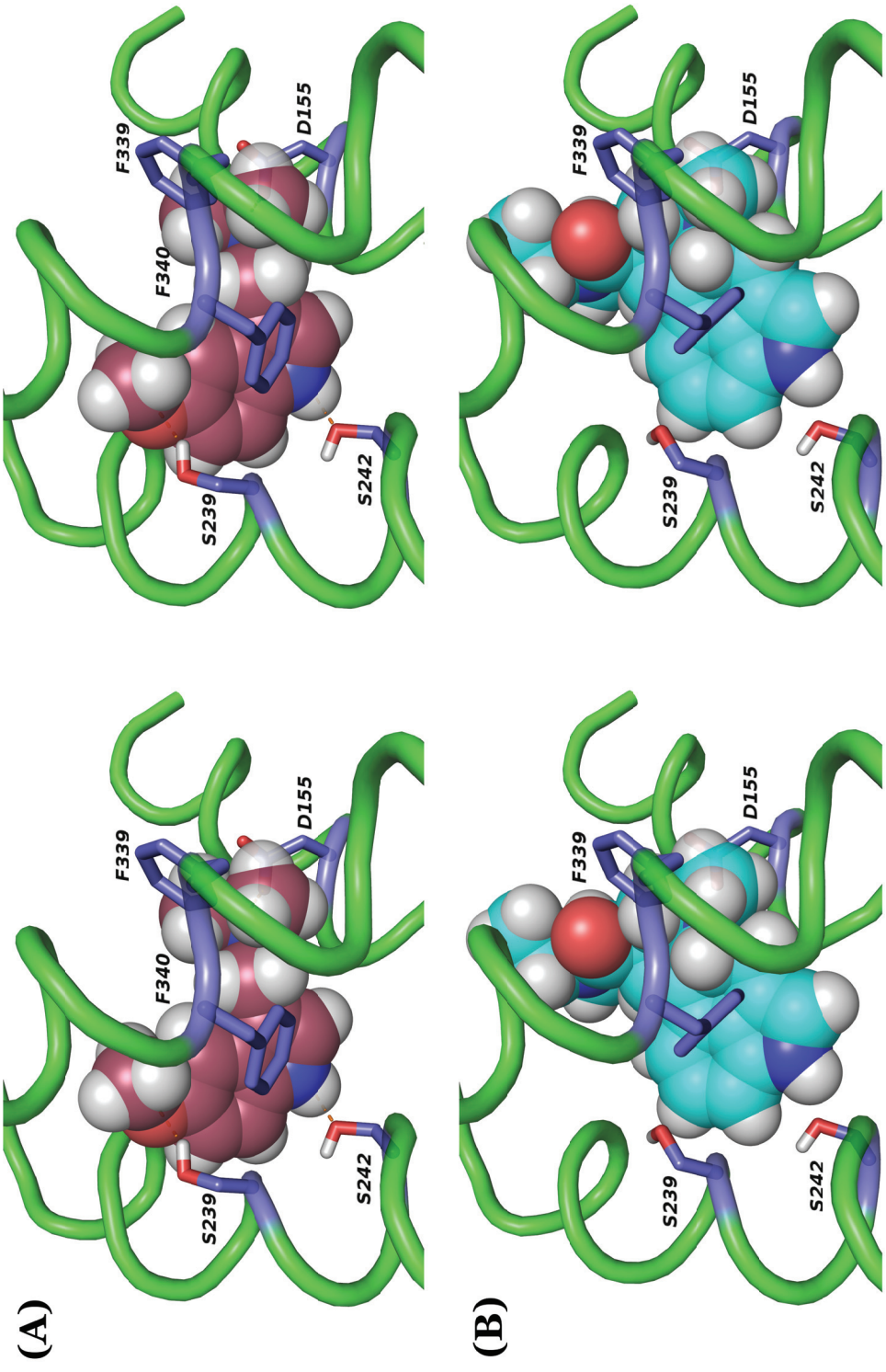

(A)
F339
F340
D155
S239
S242
F339
F340
D155
S239
S242
(B)
F339
D155
S239
S242
F339
D155
S239
S242

Figure 4.10

The S5.43(239)A mutation detrimentally affects the binding and activity of tryptamines predicted to interact with this residue

Table 4.9 and Figure 4.11 present the results of competition binding assays with wild type, S5.43(239)A, and S5.46(242)A mutant h5-HT_{2A} receptors. To aid visual interpretation, a loss in binding affinity is defined as a negative -Δ(ΔG°). Binding of tryptamines with a polar aromatic substituent, namely 5-MeO-T, 5-HT, 5-MeO-DMT, psilocin, and 1-Me-5-HT, was attenuated by the S5.43(239)A mutation to a degree consistent with the loss of a hydrogen bond (Fersht, 1988), although surprisingly, 1-iPr-5-MeO-T was relatively unaffected. Both 5-HT and 5-MeO-DMT suffered the greatest losses of affinity, about 14- and 11-fold (approximately -1.5 kcal/mol), respectively.

Table 4.10 and Figure 4.12 present measures of functional activity of the test compounds utilizing PI hydrolysis assays at wild type and mutant receptors. Generally, the trends observed in affinity changes for polar substituted tryptamines were maintained in shifts of functional potency, as defined by ΔpEC50. All compounds were affected by this mutation to a certain degree, although in some cases the effect was very slight (*e.g.* 5-MeO-T). Again, 5-MeO-DMT and 5-HT were most affected, with their potencies being reduced by about 20- and 35-fold, respectively. Psilocin was the only compound showing a statistically discernable change in intrinsic activity (about 33%), as well as showing about a 30-fold decrease in potency.

By contrast, LSD lacks a polar substituent in the area indicated by virtual docking experiments to be near S5.43(239) and, not surprisingly, this mutation had no effect on its affinity, and only a weak effect on its potency. Similarly, tryptamine and DMT also lack a polar substituent in this region; their binding was relatively unaffected and potency was only weakly affected. Although affinity of 5-Me-T also was unaffected, surprisingly we observed a dramatic 65-fold decrease in potency.

Table 4.9 Effect of the S5.43(239)A and S5.46(242)A mutations on binding to h5-HT_{2A} receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments. Except where indicated (†), mutant values of ΔpK_i were statistically distinguishable from wild-type ($p<0.05$) using ANOVA with Bonferroni post-tests comparing mutant values to wild type.

Drug	(±)-[^{125}I]DOI		
	WT K_i **(nM)**	**S239A** K_i **(nM)**	**S242A** K_i **(nM)**
LSD	0.40 (0.02)	0.41 (0.08)†	1.66 (0.32)
5-MeO-T	1.34 (0.22)	4.17 (0.68)	2.70 (0.29)
5-HT	4.84 (0.20)	53.2 (8.0)	20.2 (0.9)
5-MeO-DMT	7.54 (1.06)	105 (19)	36.0 (1.9)
5-Me-T	11.7 (0.6)	10.9 (1.3)†	25.8 (3.9)†
psilocin	11.8 (1.2)	58.0 (3.7)	23.1 (3.3)
tryptamine	29.7 (4.4)	50.2 (6.6)†	36.0 (6.0)†
1-Me-5-HT	70.0 (1.2)	320 (37)	5.73 (0.83)
DMT	75.1 (6.0)	116 (12)†	135 (20)†
1-iPr-5-MeO-T	494 (91)	786 (85)†	24.1 (0.8)

† $p > 0.05$

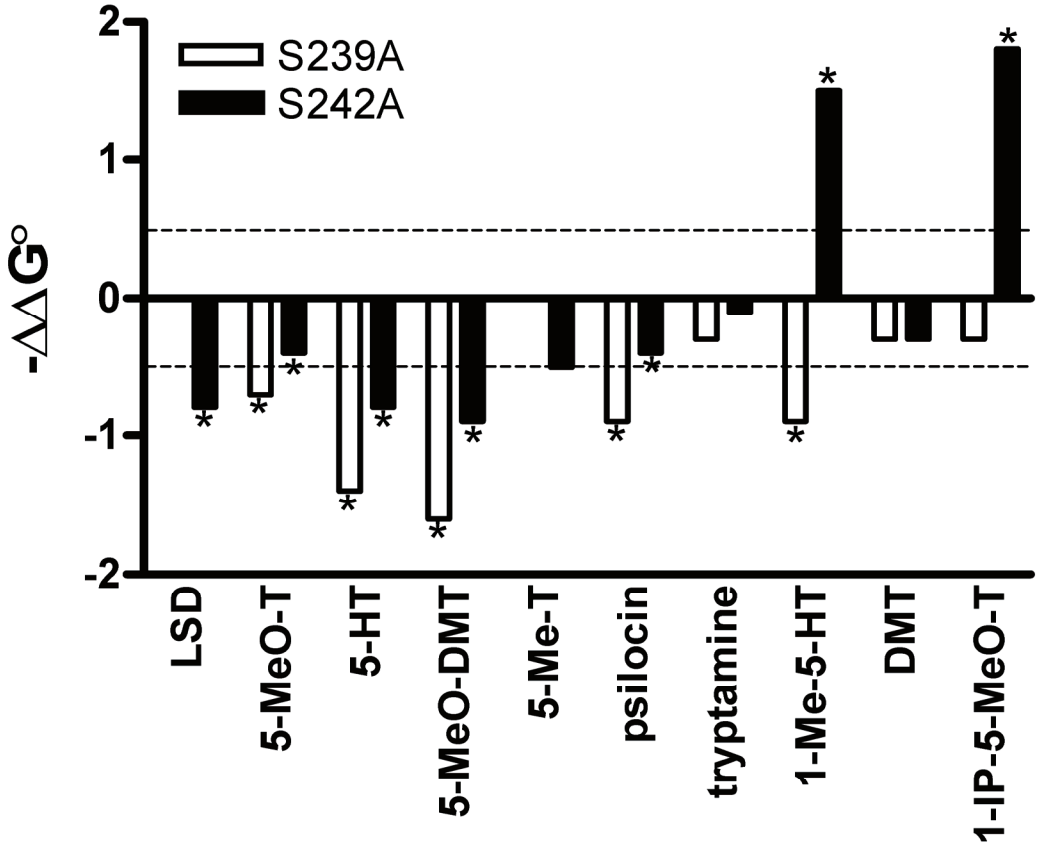


Figure 4.11 Effects of mutations of TM5 residues on the binding of tryptamines to h5-HT_{2A} receptors. Bars indicate changes in the standard Gibbs free energy of binding ($\Delta G°$) for S5.43(239)A (open bars), and S5.46(242)A (closed bars) from the data of Table 4.9. Dashed lines at ±0.5 kcal/mol indicate the lower threshold for the energetics of a hydrogen bond (Fersht, 1988). * indicates $p<0.05$ from two-way ANOVA with Bonferroni post-tests.

Table 4.10 Effect of the S5.43(239)A and S5.46(242)A mutations on h5-HT_{2A} receptor-mediated PI hydrolysis. Data are presented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity values from at least three independent experiments. Except where indicated (†), all EC50 values were statistically distinguishable from wild type as defined by $p<0.05$ from ANOVA with Bonferroni post-tests between mutant and wild type receptors; * indicates $p<0.05$ for ΔInt.Act. values using the same statistical comparison.

Drug	WT h5-HT_{2A}		S239A		S242A	
	EC50 PI Hydrolysis (nM)	**Intrinsic Activity (% 5-HT)**	**EC50 PI Hydrolysis (nM)**	**Intrinsic Activity (% 5-HT)**	**EC50 PI Hydrolysis (nM)**	**Intrinsic Activity (% 5-HT)**
LSD	0.22 (0.04)	84 (3)	0.66 (0.10)	80 (5)	1.10 (0.12)	86 (2)
5-MeO-DMT	4.3 (0.78)	98 (4)	150 (25)	88 (7)	31 (4.5)	91 (5)
5-HT	5.2 (0.97)	100	100 (8)	100	19 (3.4)	100
5-MeO-T	5.2 (0.48)	99 (4)	26 (1.2)	100 (7)	5.0 (0.45)†	100 (6)
psilocin	7.3 (0.72)	110 (9)	210 (25)	72 (8)*	45 (8.4)	92 (3)
5-Me-T	18 (3.4)	110 (8)	1200 (184)	99 (2)	21 (1.0)†	90 (3)
tryptamine	94 (18)	100 (0)	230 (41)	97 (3)	95 (17)†	96 (8)
DMT	190 (6)	70 (1)	790 (73)	91 (3)	200 (20)†	95 (4)
1-Me-5-HT	310 (30)	100 (3)	1800 (200)	101 (3)	6.9 (1.1)	95 (4)
1-iPr-5-MeO-T	2400 (180)	100 (1)	7900 (1400)	83 (8)	48 (6.7)	97 (3)

† EC50 $p > 0.05$; * Int.Act. $p < 0.05$.

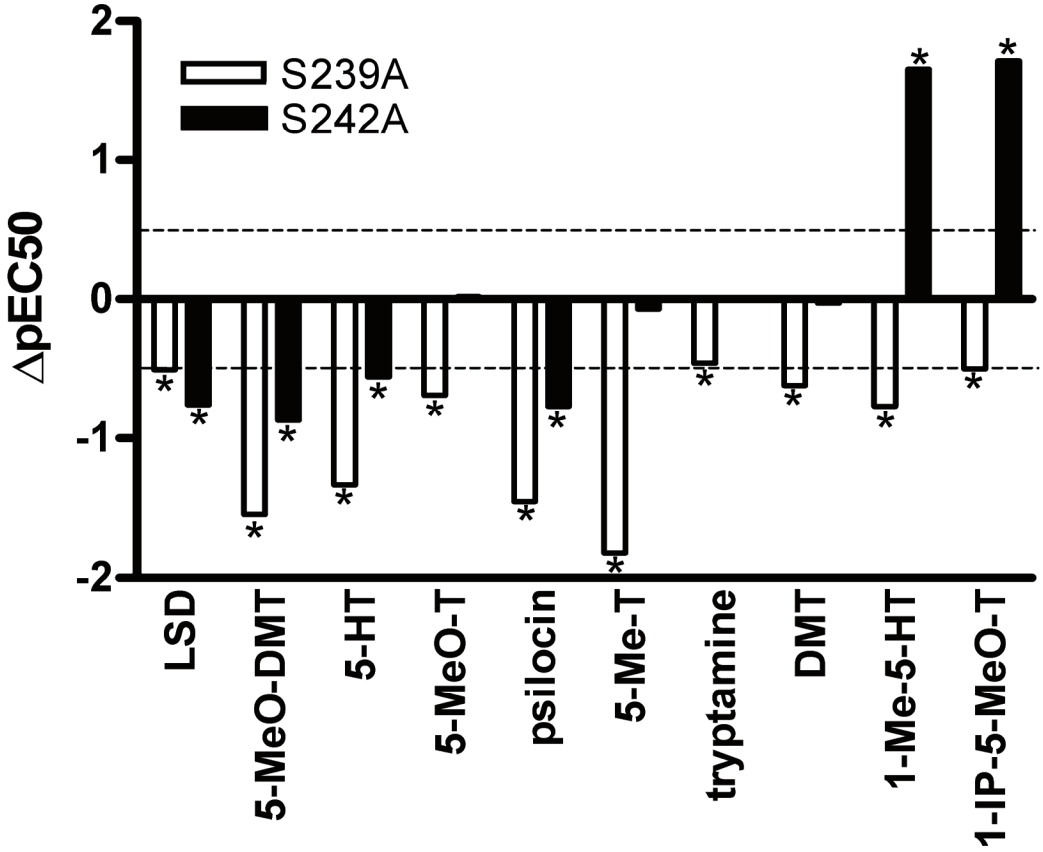


Figure 4.12 Effects of TM5 mutations on the functional potency of tryptamines at h5-HT_{2A} receptors. Bars indicate log-scale normalized changes in the functional potency (pEC50) for S5.43(239)A (open bars), and S5.46(242)A (closed bars) from the data of Table 4.10. Dashed lines at ±0.5 kcal/mol indicate an arbitrary threshold for "weak" effects. * indicates $p < 0.05$ from two-way ANOVA with Bonferroni post-tests.

The S5.46(242)A mutation in the h5-HT_{2A} receptor attenuates the affinity and activity of some tryptamines predicted to interact with this residue, while enhancing the binding and activity of tryptamines with alkyl substitution on the indole (N1) nitrogen

As Figure 4.11 illustrates, the S5.46(242)A mutation attenuated the affinity only of LSD, 5-HT, and 5-MeO-DMT to a degree consistent with the loss of a hydrogen bond (Fersht, 1988). Changes in the binding energetics of psilocin and 5-MeO-T approach the lower limit of this energy range (0.5-1.5 kcal/mol). The affinity of other tryptamines, particularly those lacking a polar ring substituent, was not significantly affected by the S5.46(242)A mutation. The tryptamines with alkyl substitution on the indole (N1) nitrogen, 1-Me-5-HT and 1-iPr-5-MeO-T, showed 12- and 21-fold *increased* affinity, respectively.

Similar trends were generally observed in functional potency, as illustrated in Figure 4.12. With the exception of 5-MeO-T, the potency of all the tryptamines with polar aromatic substituents, as well as LSD, was only weakly or moderately decreased. No shifts in potency were observed in any of the ring-unsubstituted tryptamines, namely 5-Me-T, tryptamine, or DMT. Again, we observed dramatic 45- and 51-fold increases in potency for the N(1)-alkyl analogues 1-Me-5-HT and 1-iPr-5-MeO-T, respectively.

4.3.2. Discussion

The next opportunity to investigate the fidelity of the binding site topology of our h5-HT_{2A} receptor homology model came in the characterization of tryptamines at this receptor. This specific aim focused on the interactions of tryptamines with serine residues at positions 5.43 (S5.43(239)) and 5.46 (S5.46(242)) in TM5 of the 5-HT_{2A} receptor. These two residues are located one and two turns, respectively, above the highly conserved proline residue, P5.50(246) in TM5, toward the extracellular side of the receptor. In other homologous GPCRs with a polar residue at position 5.43, mutagenesis to a non-polar residue dramatically reduced the affinity and activity of nearly all agonists tested (Strader *et al.*, 1989; Ho *et al.*, 1992; Pollock *et al.*, 1992; Wess *et al.*, 1992) or increased the affinity of antagonists and was involved in species selectivity (Link *et al.*,

1992). Mutation of a polar residue at position 5.46 in non-serotonin aminergic GPCRs also decreased the affinity of agonists (Strader *et al.*, 1989; Wang *et al.*, 1991; Pollock *et al.*, 1992), affected the selectivity of agonists or antagonists (Mansour *et al.*, 1992), and/or decreased the affinity of antagonists (Gantz *et al.*, 1992; Leurs *et al.*, 1994). Functional activity was generally unaffected (van Rhee and Jacobsen, 1996).

Previous mutagenesis studies have examined S5.43(239)A in the rat 5-HT_{2A} receptor, and the S5.46(242) in human and A5.46(242) residues in the rat 5-HT_{2A} receptors have been reciprocally mutated (Kao *et al.*, 1992; Johnson *et al.*, 1993; Johnson *et al.*, 1994; Johnson *et al.*, 1997; Shapiro *et al.*, 2000). There is some disagreement, however, between these studies as to the binding orientation of tryptamines and/or their interactions with these residues. Furthermore, these residues have not been investigated in parallel in any 5-HT_{2A} receptor.

For this specific aim, we wished to clarify the discrepancies in earlier work by mutating residues S5.43(239) and S5.46(242) to alanine in the human 5-HT_{2A} receptor and assess the effects on selected structurally-modified tryptamine derivatives. A non-disruptive deletion mutation to an alanine was chosen for each residue because it was anticipated that this change would abolish specific polar ligand-receptor interactions, but might not affect global receptor structure (Fersht *et al.* 1987). Indeed, only a 2-fold shift in K_D was observed for [^{3}H]ketanserin at S5.43(239)A receptors (1.1 vs. 2.2 nM) and a slight enhancement at S5.46(242)A receptors (1.1 vs. 0.71 nM). An approximately 3-fold shift in the K_D for [^{125}I]DOI was observed at S5.43(239)A receptors (0.64 vs. 2.19 nM) and about 2-fold at S5.46(242)A receptors (1.7 vs. 0.78 nM). The mutant S5.43(239)A and S5.46(242)A receptors still were able to induce the release of radiolabeled inositol phosphates in a dose-dependent manner that was approximately comparable in magnitude to that of the wild type receptor (data not shown), although functional potencies were slightly decreased for all chemical classes tested at the S239A receptor. Although these findings indicate that the S5.43(239)A mutation may have slightly altered G-protein coupling, we do not believe it produces a significant global disruption of receptor structure. Thus, our initial hypothesis was that any differences in ligand affinity or

activity between the wild type and S5.43(239)A or S5.46(242)A mutant receptors would result from a loss of direct interaction between the ligand and those serine residues.

The clearest results were obtained with the potent hallucinogen LSD, which was predicted not to interact with S5.43(239). The S5.43(239)A mutation had no effect at all on affinity or intrinsic activity, and only a 3-fold effect on potency (Tables 4.9 and 4.10; Figures 4.11 and 4.12). By contrast, the S5.46(242)A mutation caused a 4-fold shift in affinity (Table 4.9), resulting in a change of the standard free energy of binding ($-\Delta\Delta G°$) of -0.8 kcal/mol (Figure 4.11), within the 0.5-1.5 kcal/mol range determined for a hydrogen bond (Fersht, 1988). The S5.46(242)A mutation similarly caused an approximately 6-fold shift in potency for LSD, although it had no effect on intrinsic activity (Table 4.10 and Figure 4.12). Previous virtual docking studies in our laboratory (Chambers and Nichols 2002) oriented LSD in the putative agonist binding pocket such that there was no interaction evident with S5.43(239), whereas the indole N(1)H was indicated to engage S5.46(242), located approximately one turn below S5.43(239). The present data are consistent with this predicted binding pose. The fact that LSD is such a rigid molecule indicates that the bound ligand ensemble has little conformational freedom to involve S5.43(239) in binding. The slight loss of potency in the S5.43(239)A mutant does suggest, however, that even when a ligand does not directly engage this residue, it may still be important for secondary intramolecular interactions that create an "efficient" activated receptor state.

A second compound where there was no difference in affinity between the wild type and mutant receptors is 5-Me-T, an example of a "mutated" ligand chosen to mimic the loss of interaction between S5.43(239) and the tryptamine 5-position oxygen. We initially envisioned that addition of the 5-methyl group to tryptamine might allow a favorable Van der Waals interaction between the methyl group and A5.43(239). Indeed, although there is no difference in affinity of 5-Me-T at the wild type and S239A receptors (Figure 4.11), this ligand does have enhanced affinity at the S239A receptor compared to both 5-HT and tryptamine (Table 4.9).

Surprisingly, affinity and potency at the S5.46(242)A mutant receptor indicate that 5-Me-T does not engage this residue (Figures 4.11 and 4.12). This finding may

indicate that this ligand does not bind in an orientation similar to other tryptamines. Further confounding interpretation is the fact that although this ligand has the same intrinsic activity at the wild type and either of the two mutant receptors, there is a more than 100-fold loss of potency at the S5.43(239)A mutant receptor (Table 4.10 and Figure 4.12). Again, we believe this result suggests, as was the case for LSD, that even if an agonist ligand does not directly engage S5.43(239), this residue may still play an important indirect role in allowing the ligand-receptor ensemble to adopt an "efficient" active state.

The mutations had the most dramatic effects on the oxygen-substituted ligands 5-HT, 5-MeO-DMT, and psilocin. Although the binding energetics of psilocin were not as strongly affected, as illustrated in Figure 4.11 its functional potency was attenuated to a degree similar to the other ring-substituted tryptamines; psilocin was one of the few compounds where we observed a dramatic loss of intrinsic activity, as indicated in Table 4.10. Furthermore, with the exception of 5-MeO-T, the results support the hypothesized interaction of polar-substituted tryptamines with residue S5.43(239). These observations are consistent with the previous suggestion that 5-MeO-T may not be interacting with S5.43(239) in the rat 5-HT_{2A} receptor (Johnson *et al.*, 1997), but are in stark contrast to the conclusion that S5.43(239) is either not accessible in the binding site or is exclusively interacting with the indole nitrogen of tryptamines (Shapiro *et al.*, 2000).

The interaction of all the tryptamines with residue S5.46(242) is not so clearly evident. Indeed, it would appear that only oxygen-substituted tryptamines are detrimentally affected to a degree that would suggest loss of a hydrogen bond (Figures 4.11 and 4.12). The strong polar interaction with S5.43(239) may serve to orient the ligand, placing the indole NH in proximity to S5.46(242), where a much weaker hydrogen bond is formed. In the absence of a polar ring substituent, the indole NH interaction with S5.46(242) may be too weak, forcing those tryptamines to adopt a different binding orientation. Consistent with that argument, the ring-unsubstituted tryptamine and DMT, as well as the 5-methyl substituted 5-Me-T, were unaffected by the S5.46(242)A mutation. Moreover, the two polar substituted tryptamines with N(1)-alkylation, 1-Me-5-HT and 1-iPr-5-MeO-T, had dramatic increases in affinity and

potency at the S242A receptor compared to wild type (Figures 4.11 and 4.12). Additional support for an interaction between the tryptamine N(1)-substituent and either S5.46(242) or A5.46(242) in human or rat 5-HT_{2A} receptors, respectively, was provided by the studies of Johnson *et al.* (1994).

An earlier investigation developed a model of the rat 5-HT_{2A} receptor where both S5.43(239) and A5.46(242) were inaccessible to binding and were instead projected toward TM4 (Shapiro *et al.*, 2000). Our data are not consistent with this orientation of TM5 in the human 5-HT_{2A} receptor. Instead, we believe our data support the hypothesis that S5.43(239) is directly engaged when binding tryptamines with polar substituents at either the 4- or 5-position. Even when a direct effect cannot occur, our data suggest that residue S5.43(239) is important for activation of the 5-HT_{2A} receptor. Furthermore, polar ring-substituted tryptamines also appear to engage S5.46(242). Although the ergoline LSD lacks a polar substituent to interact with S5.43(239), it has other structural features that may orient it within the binding site, so that it can engage S5.46(242). Tryptamines lacking polar ring substituents do not appear to interact with this residue, and may be adopting an alternate binding orientation.

Our results also are consistent with the role of S5.43(239) as a hydrogen bond donor, whereas it seems more likely that S5.46(242) serves as a hydrogen bond acceptor. This idea is reinforced by the presence of alanine at position 5.46 in the human 5-HT_{2B}, 5-HT_{2C}, and 5-HT_{1A}, as well as the rat 5-HT_{2A} receptors. Further, our modeling of the h5-HT_{2A} receptor suggests that S5.46(242) may form an intrahelical hydrogen bond to the backbone carbonyl of G5.42(238). From a mechanical point of view, if P5.50(246) serves to allow the top of TM5 to undergo movement upon ligand binding, engaging a polar residue more distal from P5.50(246) would provide greater mechanical force to displace the helix, relative to a residue closer to P5.50(246).

Our data also indicate that not all tryptamines interact with the receptor in the same way, a conclusion reached by previous studies based on interactions with residue S5.43(239) in the rat 5-HT_{2A} receptor (Johnson *et al.*, 1997). The present results extend previous investigations into the role of these residues in the h5-HT_{2A} receptor. As with investigations by our laboratory into the aromatic contacts of TM6 discussed in Specific

Aim 2, it appears that the topology of the receptor binding site is such that a contact with TM5 is necessary for high affinity and functional potency of agonists. Finally, these results further support the topology and utility of our *in silico*-activated homology model of the h5-HT_{2A} receptor.

4.4. Interactions of phenylalkylamines with residues in TM5 of the h5-HT_{2A} receptor

The primary goal of this specific aim was to explore further the topology of our h5-HT_{2A} receptor homology model. These investigations focused on providing empirical evidence for the orientations of phenylalkylamines observed in virtual docking simulations. Most studies investigating binding orientations and receptor binding site residues of 5-HT_{2A} receptors have focused primarily on the ergoline and tryptamine class. This emphasis is understandable, as these ligands are structurally related to the endogenous tryptamine ligand serotonin (see Introduction).

Little empirical evidence exists for the predicted interactions of phenylalkylamines in the putative binding site of 5-HT_{2A} receptors. As discussed in the introduction and in Specific Aims 1 and 2, virtual docking simulations of phenylalkylamines to our h5-HT_{2A} receptor homology model indicate a number of contacts with polar and aromatic residues of TM3, 5, and 6. Of particular relevance to this specific aim, docking studies consistently orient the 5-methoxy oxygen of ring-substituted phenylalkylamines in proximity to S5.43(239). As there is previous work to support a key interaction of tryptamines with this residue, including Specific Aim 3, it was hypothesized that a key hydrogen bond interaction between S5.43(239) and the 5-oxygen of phenylalkylamines occurs for their binding and activity as well. In contrast to the tryptamines, however, no polar substituents of the phenylalkylamines were observed in proximity to Ser5.46(242) and thus it was predicted that no direct interaction occurs between this residue and the phenylalkylamines. Finally, these orientations generally placed the alkyl or halogen 4-substituent in proximity to Gly5.42(238). Site-directed mutagenesis of Gly5.42(238), Ser5.43(239), and Ser5.46(242), as well as reciprocal alteration of ligand structure, were utilized to investigate these hypotheses.

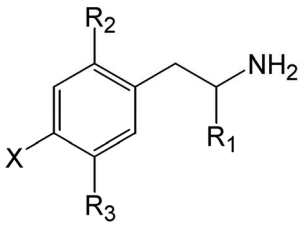
4.4.1. Results

The human G5.42(238)A mutant 5-HT_{2A} receptor possess acceptable affinity and expression levels

Results of saturation binding experiments at wild type, S5.43(239)A and S5.46(242)A mutant receptors are presented in the results of Specific Aim 3. Saturation isotherm binding assays with G5.42(238)A mutant h5-HT_{2A} receptors utilizing [^{3}H]ketanserin and [^{125}I]DOI yielded K_D values of 3.66 ± 0.46 nM and 0.87 ± 0.03 nM, respectively. Receptor expressions based on B_{max} values from [^{3}H]ketanserin saturation binding assays were 1838 ± 339 fmol/mg

Virtual docking of phenylalkylamines to an *in silico*-activated h5-HT_{2A} receptor homology model indicate that S5.43(239) interacts with the 5-oxygen of the ligand, whereas S5.46(242) does not interact with this ligand class

Figure 4.14 illustrates a representative binding pose for virtual docking and subsequent energy minimization simulations of phenylalkylamines to an *in silico*-activated homology model of the h5-HT_{2A} receptor. We observed final low energy ensemble binding poses for the phenylalkylamines that position their aromatic substituents in regions and orientations able to interact with h5-HT_{2A} receptor residues. Of particular relevance for this aim, and as predicted previously (Chambers and Nichols, 2002), the 5-methoxy of phenylalkylamines is positioned to indicate possible hydrogen bonding with S5.43(239). Virtual docking simulations do not, however, indicate any interaction between S5.46(242) and phenylalkylamines. By contrast, S5.46(242) may interact with tryptamines, as reported by others (Kao *et al.*, 1992; Johnson *et al.*, 1994), and in Specific Aim 3. These orientations also appear to place the 4-position substituents of phenylalkylamines in proximity to Gly5.42(238).



	X	R_1	R_2	R_3
2CH	H	H	OCH_3	OCH_3
2CI	I	H	OCH_3	OCH_3
DOH	H	CH_3	OCH_3	OCH_3
DOI	I	CH_3	OCH_3	OCH_3
DOM	CH_3	CH_3	OCH_3	OCH_3
2-Et-DOM	CH_3	CH_3	CH_2CH_3	OCH_3
5-Et-DOM	CH_3	CH_3	OCH_3	CH_2CH_3
5-H-DOM	CH_3	CH_3	OCH_3	H

Figure 4.13 Structures of phenylalkylamines used for Specific Aim 4

Figure 4.14 Illustrative cross-eyed stereopair representation of ligand poses from virtual docking experiments with **(A)**, (*R*)-DOM, and **(B)**, 2CH, in the h5-HT_{2A} receptor showing predicted polar interactions between the ligand and receptor residues. Ligands are shown as space-filling spheres, whereas receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM5 on the left, TM6 in the right foreground, TM3 in the right background, and the extracellular face of the receptor toward the top of the figure. TMs 1, 2, 4, and 7 are not displayed.

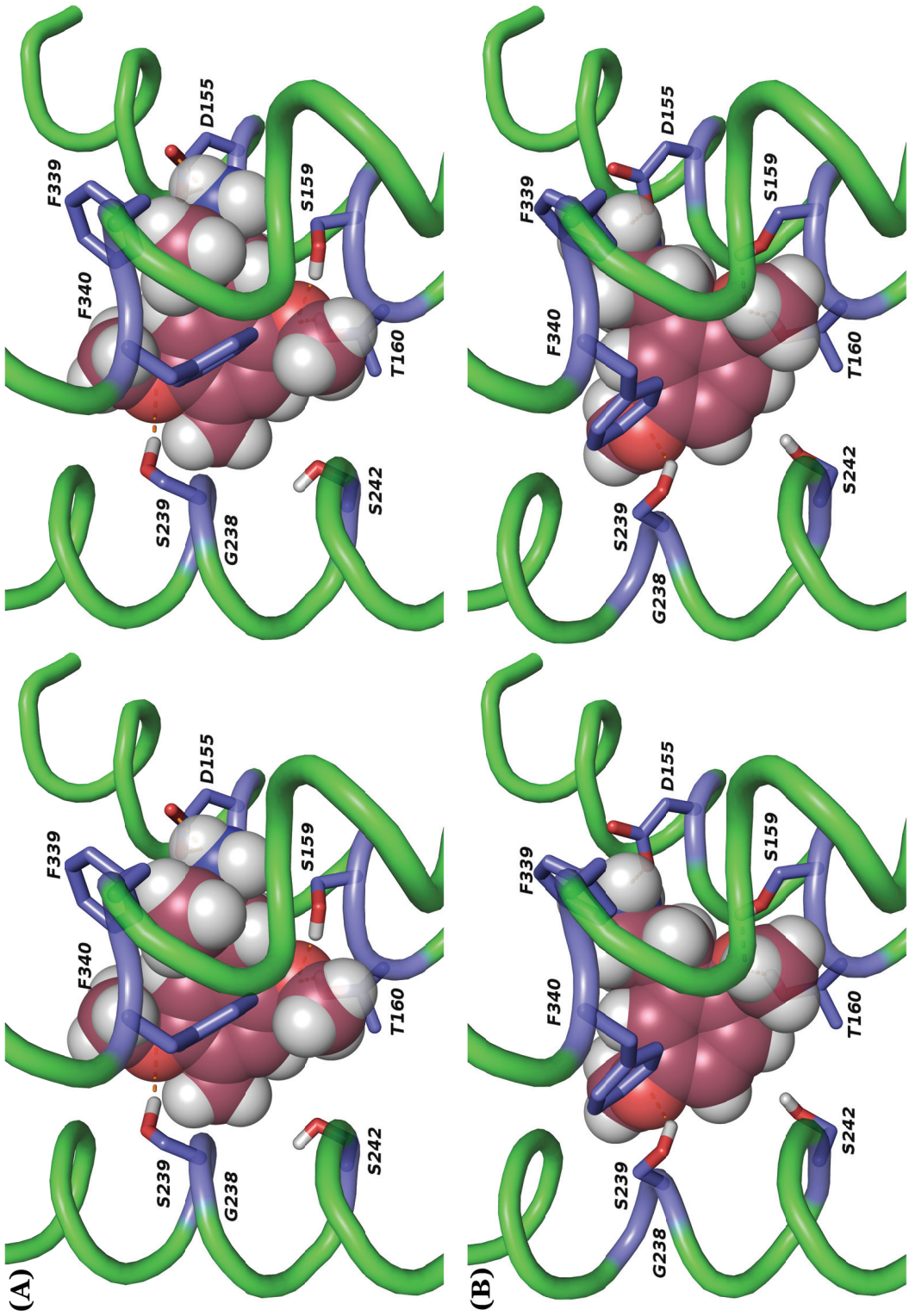


Figure 4.14

Table 4.11 Effect of the G5.42(238)A, S5.43(239)A, and S5.46(242)A mutations on binding to h5-HT_{2A} receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments. * indicates values statistically distinguishable from wild-type, with $p<0.05$ for values of ΔpK_i obtained by ANOVA with Bonferroni post-tests comparing mutant values to wild type.

Drug	(±)-[^{125}I]DOI			
	h5-HT_{2A} K_i (nM)	**G238A K_i (nM)**	**S239A K_i (nM)**	**S242A K_i (nM)**
DOI	0.64 (0.06)	0.64 (0.09)	2.19 (0.26)*	0.83 (0.11)
2CI	0.73 (0.06)	0.74 (0.08)	1.20 (0.22)	1.28 (1.28)
DOM	5.91 (0.97)	3.73 (0.59)	46.4 (5.9)*	5.04 (0.55)
5-Et-DOM	22.4 (1.2)	43.3 (0.8)	32.2 (1.9)	31.5 (5.0)
2-Et-DOM	91.1 (9.7)	44.2 (8.4)	460 (92)*	38.5 (4.1)
5-H-DOM	169 (12)	108 (14)	343 (41)	164 (7)
DOH	245 (28)	30.0 (2.4)*	2250 (140)*	294 (54)
2CH	377 (67)	43.7 (7.9)*	782 (19)	440 (23)
isoproscaline	465 (66)	2290 (380)*	1630 (200)*	157 (26)*
escaline	611 (72)	853 (31)	526 (57)	109 (33)*
mescaline	1500 (240)	692 (54)	2020 (380)	456 (58)*

* $p < 0.05$

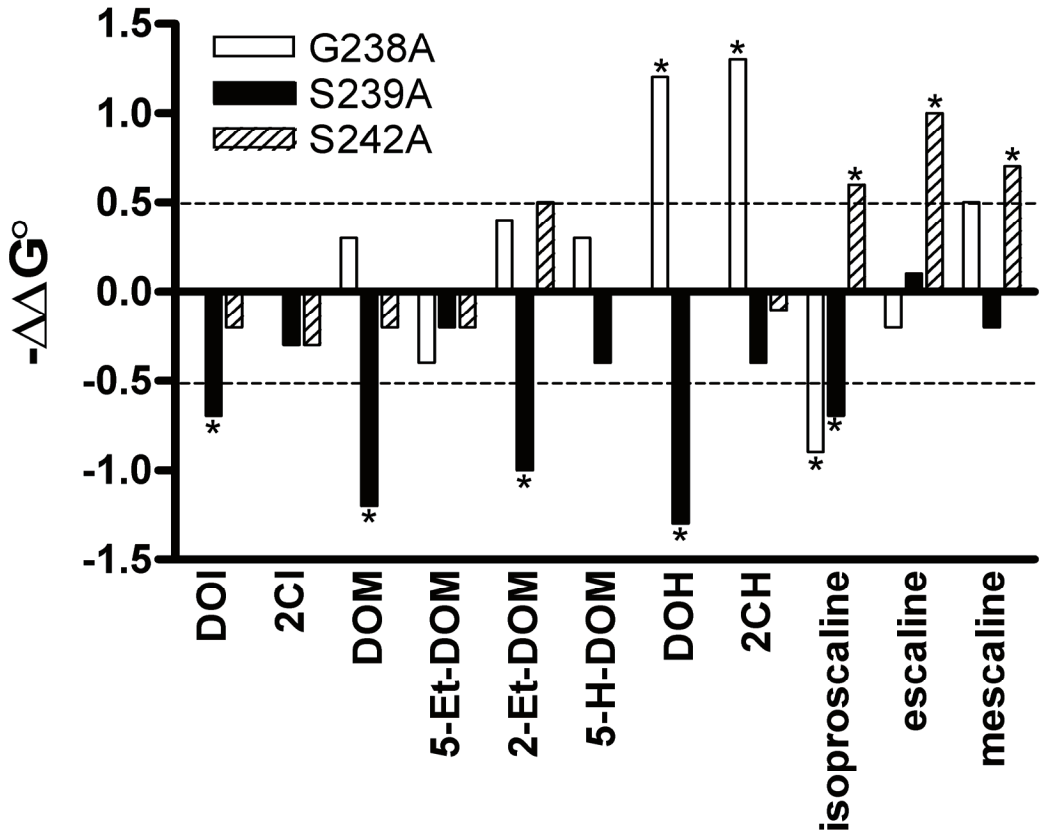


Figure 4.15 Effects of mutation of TM5 residues on the binding of phenylalkylamines to h5-HT_{2A} receptors. Bars indicate changes in the standard Gibbs free energy of binding ($\Delta G°$) for G5.42(238)A (open bars), S5.43(239)A (closed bars), and S5.46(242)A (shaded bars) from the data of Table 4.11. Dashed lines at ±0.5 kcal/mol indicate the lower threshold for the energetics of a hydrogen bond (Fersht, 1988). * indicates $p < 0.05$ generated from two-way ANOVA with Bonferroni post-tests.

Table 4.12 Effects of the G5.42(238)A, S5.43(239)A, and S5.46(242)A mutations on h5-HT_{2A} receptor-mediated PI hydrolysis. Data are presented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity values from at least three independent experiments. A typical experiment would show 4-10 fold stimulation by 5-HT over basal at all receptors. All raw counts were normalized to basal (0%) and serotonin (100%). Except where indicated (†), all EC50 values were statistically distinguishable from wild type as defined by $p<0.05$ from ANOVA with Bonferroni post-tests between mutant and wild type receptors; * indicates $p<0.05$ values for ΔInt.Act. values using the same statistical comparison.

Drug	h5-HT_{2A}		G238A		S239A		S242A	
	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)
DOI	3.79 (0.36)	95 (7)	4.22 (0.53)†	99 (9)	38.9 (8.9)	92 (12)	1.37 (0.06)	92 (2)
DOM	2.81 (0.22)	87 (3)	16.8 (2.9)	87 (2)	620 (103)	87 (3)	6.46 (1.23)	91 (2)
2CI	4.81 (0.89)	87 (7)	8.45 (1.46)†	95 (6)	71.8 (13.6)	52 (5)*	5.93 (0.58)†	86 (3)
2-Et-DOM	49.8 (7.0)	81 (2)	126 (25)	93 (6)	2975 (546)	75 (6)	22.6 (2.8)	93 (2)
5-Et-DOM	71.9 (10.8)	84 (10)	131 (27)†	101 (5)	571 (78)	81 (6)	62.0 (10.8)†	89 (7)
DOH	284 (22)	104 (5)	94.0 (16.8)	93 (3)	16244 (883)	100 (2)	1793 (298)	101 (7)
5-H-DOM	533 (64)	106 (5)	260 (16)†	91 (1)	2532 (108)	98 (1)	245 (40)†	98 (1)
2CH	1021 (14)	96 (10)	453 (88)	99 (5)	13053 (416)	100 (4)	4917 (346)	92 (5)
isoproscaline	220 (34)	101 (3)	6289 (281)	94 (5)	5336 (1017)	92 (7)	48.3 (6.9)	96 (6)
escaline	275 (55)	100 (3)	3000 (118)	102 (6)	4468 (849)	97 (3)	61.1 (10.74)	96 (3)
mescaline	1117 (223)	83 (5)	3152 (361)	96 (8)	5825 (1007)	79 (6)	608 (79)†	94 (2)

† EC50 $p > 0.05$; * Int.Act. $p < 0.05$

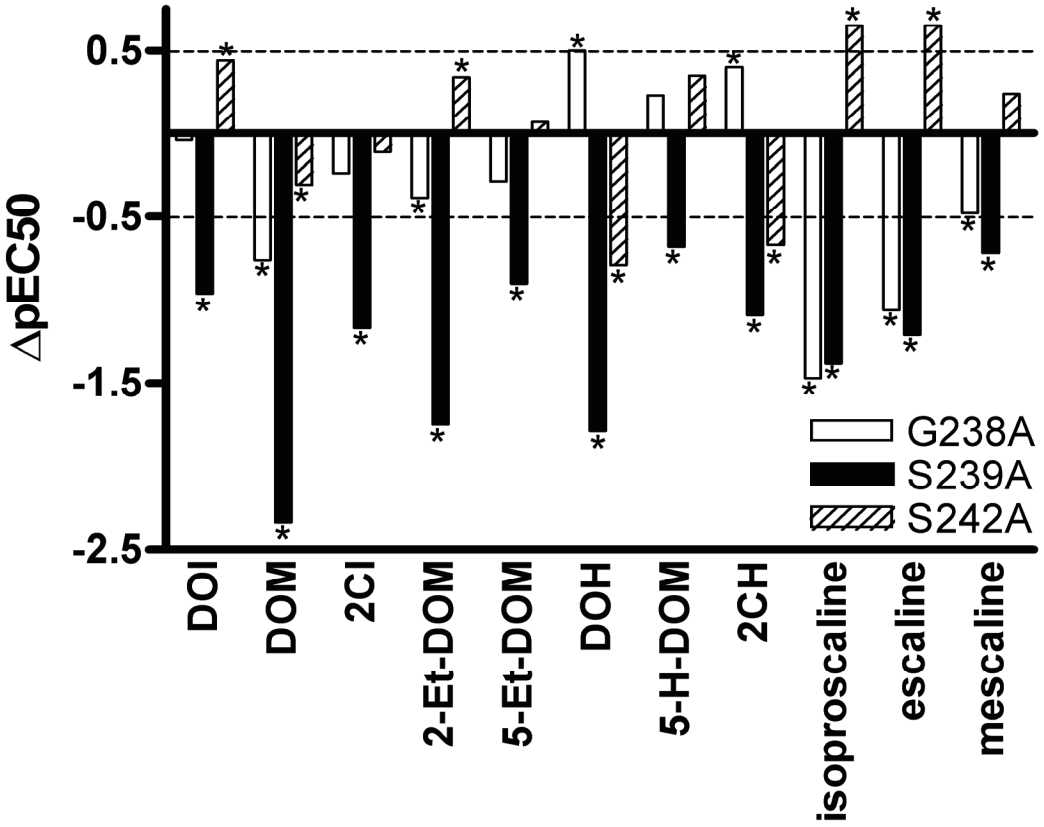


Figure 4.16 Effect of mutations of TM5 residues on the functional potency of phenylalkylamines at h5-HT_{2A} receptors. Bars indicate log-scale normalized changes in the functional potency (pEC50) for G5.42(238)A (open bars), S5.43(239)A (closed bars), and S5.46(242)A (shaded bars) from the data of Table 4.12. Dashed lines at ±0.5 kcal/mol indicate an arbitrary threshold for "weak" effects. * indicates $p < 0.05$ generated from two-way ANOVA with Bonferroni post-tests.

The S5.43(239)A mutation in the h5-HT_{2A} receptor detrimentally affects the binding and activity of most phenylalkylamines

Table 4.11 and Figure 4.15 present the results of competition binding assays with wild type and mutant h5-HT_{2A} receptors. Again, the energetic contribution to the standard Gibbs free energy of binding ($\Delta G°$) of a hydrogen bond is generally believed to be between 0.5-1.5 kcal/mol (Fersht, 1988). Thus, the differences in binding energy of this magnitude between wild type and mutant receptors ($\Delta\Delta G°$) illustrated in Figure 4.15 are consistent with the loss of a hydrogen bond. As with the previous figures, to aid visual interpretation, negative values displayed in Figure 4.15 connote a loss in binding affinity due to the mutation.

The affinities of the phenylisopropylamines DOI, DOM, and DOH were detrimentally affected by the S5.43(239)A mutation, with $\Delta\Delta G°$ well within the range given above for hydrogen bonds. The phenethylamines 2CI and 2CH also were detrimentally affected, but the $\Delta\Delta G°$ values were less than 0.5 kcal/mol, and not statistically discernable from wild type. Mescaline and its ethoxy analogue escaline were unaffected, but its isopropoxy analogue isoproscaline was perturbed to a moderate degree.

Effects on functional potency were measured by changes in EC50 (ΔpEC50), and effects on efficacy were measured by changes in intrinsic activity (ΔInt.Act.), as presented in Table 4.12 and illustrated in Figure 4.16. Again, a loss in functional potency or intrinsic activity due to the mutation is indicated by a negative value.

All compounds tested showed significant decreases in potency (ΔpEC50) at the S5.43(239)A mutant receptor, compared to wild type. DOM and DOH were the most markedly affected ligands, with decreases of about 60- and 220-fold, respectively. Of all the compounds tested at all the mutant receptors in this specific aim, we observed a decrease in intrinsic activity only with 2CI at h5-HT_{2A}/S5.43(239)A receptors (ΔInt. Act. $\approx$ -35%).

A complementary alteration of ligand structure can produce binding and activity changes at the WT h5-HT_{2A} receptor similar to those of the unmodified ligand at the h5-HT_{2A}/S239A receptor, and can rescue some binding and activity at the S5.43(239)A mutant receptor

As Table 4.13 indicates, removal of a polar group from the aromatic ring of the phenylisopropylamine DOM to yield 2-Et-DOM, 5-Et-DOM, or 5-H-DOM gave changes in affinities at the wild type receptor consistent with the energetics of a lost hydrogen bond (Fersht, 1988). These shifts are similar in magnitude to those observed when comparing the polar-substituted ligand homologues in wild type versus S5.43(239)A mutant receptors, such as the effect of the S5.43(239)A mutation on DOI or DOM in Figure 4.15. Removal of the polar group at the 5-position of DOM to give 5-Et-DOM did not detrimentally affect binding at the S5.43(239)A mutant receptor. This mutant was still sensitive to the complete removal of the 5-methoxy to give 5-H-DOM ($\Delta\Delta G°$ -1.2), although it was not, however, as sensitive as the wild type receptor ($\Delta\Delta G°$ -2.0).

Table 4.13 Effects of change in ligand structure on binding affinity and functional potency at wild type and S5.43(239)A mutant h5-HT_{2A} receptors. Data are presented as $-\Delta\Delta G°$ or ΔpEC50 values based on data from Tables 4.11 and 4.12. Negative values indicate a loss in affinity or functional potency due to the change in ligand structure. Values around 0 indicate that the receptor is insensitive to the change of ligand structure being compared. Except where indicated (†), all values for $-\Delta\Delta G°$ or ΔpEC50 generated $p<0.05$ from unpaired two-tailed Student T-tests between ligands at the particular receptor compared (column heading).

Drug	$-\Delta\Delta G°$		ΔpEC50	
	h5-HT_{2A}	S239A	h5-HT_{2A}	S239A
DOM vs. 2-Et-DOM	-1.6	-1.4	-1.2	-0.7
DOM vs. 5-Et-DOM	-0.8	0.2†	-1.4	0.0†
DOM vs. 5-H-DOM	-2.0	-1.2	-2.3	-0.6

† $p > 0.05$

Table 4.13 also illustrates the difference in functional potency between wild type and S5.43(239)A mutant receptors when a polar group is removed from phenylisopropylamine ligands. Similar to the binding energies, the functional potencies at the h5-HT_{2A} wild type receptor are sensitive to the removal of a polar group either at the 2- or 5-position of phenylalkylamines. By contrast, the S5.43(239)A mutant receptor is generally less sensitive or even insensitive to the loss of a polar group, but only at the ligand 5-position. This insensitivity is clearly evident in the comparison of DOM to 5-Et-DOM. Although we did not expect 2-Et-DOM and DOM to have significantly different potencies at the h5-HT_{2A}/S239A mutant receptor, the loss of sensitivity to substituting the 2-oxygen (2-Et-DOM; ΔpEC50 1.2 vs. 0.7) is not as great as seen with either substitution (5-Et-DOM; ΔpEC50 1.4 vs. 0.0), or removal (5-H-DOM; ΔpEC50 2.3 vs. 0.6) of the 5-oxygen substituent.

The S5.46(242)A mutation in the h5-HT_{2A} receptor does not detrimentally affect the binding and activity of most phenylalkylamines

As Table 4.11 indicates, the S5.46(242)A mutation did not detrimentally affect the binding of any of the compounds tested to a degree statistically discernable from wild type. This result is consistent with our observation that there is no significant difference between the affinity of phenethylamine agonists at the human and rat 5-HT_{2A} receptors (see Specific Aim 1), which differ mainly in that the human receptor binding site has a serine at this position, whereas the rat receptor has an alanine. Interestingly, mescaline and its analogues had increased binding affinity at this mutant receptor. This relationship is illustrated in Figure 4.15.

As Table 4.12 indicates, at the S5.46(242)A mutant receptor DOM, DOH, and 2CH suffered generally weak losses in potency of 2-, 6-, and 5-fold, respectively, whereas the potency of DOI was slightly increased, about 3-fold. Mescaline and its analogues also had increased potency due to this mutation of about 2- to 4-fold. The S5.46(242)A mutation did not lead to a significant change in efficacy for any of the compounds tested. No compounds had intrinsic activities altered to a degree discernable from wild type. This relationship is illustrated in Figure 4.16.

The G5.42(238)A mutation in the h5-HT_{2A} receptor affects some phenylalkylamines in a manner dependent on the presence or size of their 4-substituent

As Table 4.11 indicates, the G5.42(238)A mutation enhanced the binding affinity by 8- and 9-fold, respectively, only of the two phenylalkylamines tested that lacked substituents in the 4-position, DOH and 2CH. Only isoproscaline, with a large 4-isopropoxy group, had a decrease of affinity of about 5-fold. No other compounds had their affinity affected by this mutation to a degree statistically discernable from wild type. This relationship is illustrated in Figure 4.15.

As Table 4.12 indicates, the functional potencies of the compounds lacking a 4-substituent, DOH and 2CH, were weakly enhanced, about 3-fold each, at the G5.42(238)A mutant receptor. This mutation detrimentally affected the potencies of DOM (6-fold) and 2-Et-DOM (2-fold). The other phenylalkylamines with 4-substitution, namely DOI, 2CI, and 5-Et-DOM had slight decreases in their potencies, but these were not statistically distinguishable from wild type. The degree to which mescaline and its analogues were affected appeared to depend on the size of their 4substituent; that is, mescaline had a 5-fold decrease in potency, escaline a 16-fold decrease, and isoproscaline a 24-fold decrease. This relationship is illustrated in Figure 4.16. No compounds had any changes in intrinsic activity that were statistically distinguishable from wild type.

4.4.2. Discussion

Having gained insight into the binding mode(s) of tryptamine agonists in Specific Aim 3, we wished to understand how the structurally-unrelated phenethylamines bound to this receptor, particularly with regard to the positioning of aromatic groups. Extensive structure-activity relationship studies of the phenylalkylamine pharmacophore have revealed the optimal placement of ring substituents: methoxy groups at the 2- and 5-positions, and a non-polar moiety such as a halogen, aliphatic alkyl, or alkylthio group in the 4-position (Nichols, 1981; Glennon *et al.*, 1986; Glennon, 1989; Shulgin and Shulgin, 1991; Nichols, 1994; Nichols, 1997; Glennon, 1999). We reasoned that the present specific aim would help to identify the basis for these molecular features. We were particularly interested in the hypotheses, as exemplified in Figure 4.14, that: (1)

S5.43(239) is critical for engaging the polar 5-position oxygen; (2) this ligand orientation places the non-polar 4-position group in proximity to G5.42(238); and (3) S5.46(242) is not positioned to interact with any phenylalkylamine's polar groups.

As with Specific Aim 3, mutation to an alanine residue was chosen for the serine residues because it was anticipated that this change would disrupt specific ligand-receptor interactions without a dramatic perturbation of global receptor structure (Fersht *et al.* 1987). Similarly, a non-disruptive addition mutation to alanine was chosen for the glycine residue, as it was anticipated that the additional steric bulk might affect the binding of phenylalkylamines with different 4-position substituents. As Tables 4.11 and 4.12 indicate, each mutant receptor shows the ability to bind and/or displace sufficient radiolabeled ligand and to induce the hydrolysis of radiolabeled phosphatidylinositides (PI) in a dose-dependent manner. Furthermore, slight changes in ligand structure produced different magnitudes of effects in both the binding and functional assay systems. Thus, a dramatic perturbation of receptor structure was not observed and changes in ligand structure could further be used to probe the sensitivities of the receptors to the presence or absence of groups attached to different sites in the ligand molecules.

Indeed, this use of "mutated" ligands and shifts in sensitivity indicated by Table 4.13 yields the most compelling evidence for the interaction of S5.43(239) with the phenylalkylamines, particularly the phenylisopropylamine subclass. At the wild type receptor, one clearly observes the necessity of the polar oxygen in the ligand 5-position, reflected in the sensitivity to its removal from DOM in the 5-ethyl or 5-hydrogen homologues 5-Et-DOM and 5-H-DOM, respectively. Moreover, the binding energetics and functional potency shifts at the S5.43(239)A mutant receptor indicate insensitivity to the absence of a 5-oxygen atom in 5-Et-DOM, the most direct 5-desoxy homologue of DOM. The S5.43(239)A mutant receptor is not completely insensitive to this change, as seen with 5-H-DOM, and also have reduced sensitivity to 2-Et-DOM. The magnitude of sensitivity loss is greatest, however, with the 5-position homologues. That 5-H-DOM has lower affinity and potency than DOM in the S5.43(239)A mutant receptor should not be surprising, as there are likely entropic and/or solvation factors with the removal of so many ligand atoms. A cartoon of this interaction is provided in Figure 4.17.

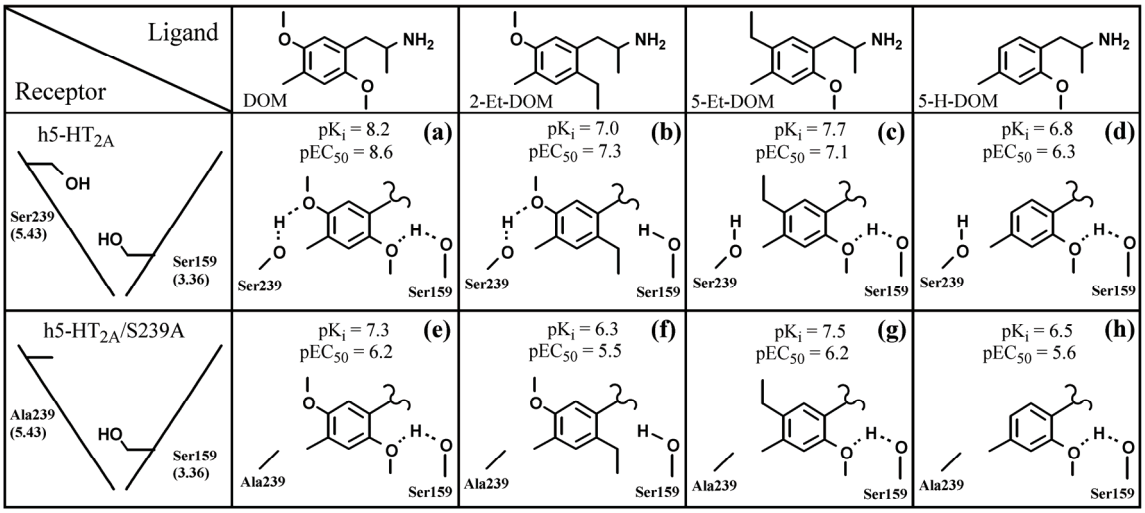


Figure 4.17 Cartoon illustration of effects of the S5.43(239)A mutation and reciprocal alteration of ligand structure.

As illustrated in Figure 4.15, the classic phenylalkylamines affected by the S5.43(239)A mutation were limited to the phenylisopropylamine subclass, namely DOI, DOM, and DOH. This trend is maintained in the functional data, as illustrated in Figure 4.16. One observes, however, that even though an interaction with the phenethylamines 2CI and 2CH is not apparent in the affinity assays, a moderate effect on the functional potency of these compounds is observed at the S5.43(239)A receptor.

Compared to phenethylamines, the phenylisopropylamines DOI and DOM have additional steric bulk on the alpha carbon that can alter their conformation. We hypothesized that the S5.43(239)A mutation would eliminate the favorable interaction with the 5-methoxy of these ligands. Not surprisingly, we observed a 3-fold and nearly 8-fold loss of affinity at the mutant receptor for DOI and DOM, respectively, compared to the wild type (Table 4.11). This difference can be related to the nature of the non-polar 4-substituent, which is known to affect both affinity and potency (Nichols and Glennon, 1984); that is, DOI has nearly 10-fold higher affinity than DOM in the wild type receptor. Hence, the 4-iodo substituent adds binding energy, probably through a Van der Waals interaction, so that the affinity of DOI is less affected by the S5.43(239)A mutation than DOM. A parallel to this reasoning in seen in the comparison of the potencies of these two ligands, as illustrated in Figure 4.16. The potency of DOM in the

S5.43(239)A mutant receptor is reduced more than 200-fold, whereas the potency of DOI is reduced only about 10-fold. DOM also is one of the few compounds that is less potent in the G5.42(238)A mutant (discussed below).

The phenethylamines 2CI and 2CH also were predicted to interact with S5.43(239), yet the changes in energetics of binding are not consistent with the range of energy for a hydrogen bond (Figure 4.15; Fersht 1988). As Figure 4.16 illustrates, however, the losses in potency due to the S239A mutation surpassed those of DOI. In addition, 2CI was the only compound that had significant and dramatically decreased intrinsic activity in the S5.43(239)A mutant. The reduced restraints on conformation or binding orientation of these compounds may allow for degenerate binding states, whereas the functionally potent active state still requires an interaction with S5.43(239). Moreover, it is apparent that the addition of the alpha-methyl group in DOI must somehow be compensating for the loss of S5.43(239), perhaps by a hydrophobic interaction of the alpha-methyl of DOI with a hydrophobic residue in TM6, F6.51(339) for example (see Specific Aim 1).

Virtual docking studies had suggested that the 4-substituent of the phenethylamines was located in the vicinity of G5.42(238). Thus, it was hypothesized that a G5.42(238)A mutation would add steric bulk to the receptor in this region that might detrimentally affect the binding and activity of phenylalkylamines with large 4-substituents, *e.g.* DOI or 2CI. Although the affinities of these 4-iodo and the 4-methyl (DOM) homologues were unaffected, and functional potency was mostly unaffected by the G5.42(238)A mutation, there is some gain of affinity and potency when the 4-substituent is removed, as in DOH and 2CH. We speculate that the methyl group of the alanine in the mutant receptor may substitute for the absence of a 4-substituent on the phenylalkylamines and allow tighter packing of these weakly potent ligands into the receptor. Also consistent with our hypothesis, the functional potency of both DOM and 2-Et-DOM was slightly decreased in this mutant. Mescaline and its analogues were affected, particularly in their functional potency, in direct relation to the size of their 4-position substituent. These findings seem to support the idea that residue G5.42(238) may be located near the 4-position of the phenylalkylamines. We speculate that a residue

larger than glycine may not be tolerated at position 5.42 in order to leave a volume to fit the spatially larger tryptamine moiety of serotonin, when compared to the smaller phenethylamines. To study the effect of an even larger residue at position 5.42 we did create a stable G5.42(238)V mutant receptor. Although we were able to label the receptor with [^{3}H]-ketanserin, the binding of [^{125}I]-DOI was too variable to determine a consistent K_d or B_{max} (data not shown), and we were not able to use this mutant for further studies.

Finally, and as anticipated, the S5.46(242)A mutation had no effect on the binding of any of the ligands tested. This result contrasts with the results for tryptamines, described in the previous specific aim. The functional potencies of DOH and 2CH were decreased, but this effect was weak to moderate and no other compounds tested were affected. The lack of 4-substitution in these compounds may allow for more conformational freedom and possibly additional binding orientations/degenerate binding states. Generally, and as hypothesized, it appears that S5.46(242) does not interact with phenylalkylamine ligands in a manner that affects binding to or activation of the h5-HT_{2A} receptor.

We believe these data support the hypothesis that S5.43(239) is directly engaged when binding most phenylalkylamines with a polar aromatic substituent in the 5-position. The interaction appears particularly strong for phenylisopropylamines. Even when a direct interaction appears unlikely, our data are consistent with the importance of S5.43(239) in the activation of the 5-HT_{2A} receptor. These results support and extend previous investigations of this residue in the 5-HT_{2A} receptor to explain more thoroughly the molecular interactions of phenylalkylamines with this receptor. We further believe that these data clearly illustrate the lack of any interaction of this class of ligands with S5.46(242), as well as providing evidence for the proximity of G5.42(238) to the 4-substituent of phenylalkylamine ligands. In total, these results are consistent with the topology of our *in silico*-activated homology model of the h5-HT_{2A} receptor.

4.5. Hydrogen bond interactions of phenylalkylamines with polar residues in TM3 of the h5-HT_{2A} receptor

The primary goal of this specific aim was to explore further the topology of our h5-HT_{2A} receptor homology model as it relates to phenylalkylamine docking orientations from virtual docking simulations. This aim focused on interactions of the polar substituents on the aromatic ring of phenylalkylamines with polar residues in TM3 of the h5-HT_{2A} receptor. Ergolines, such as LSD, lack these polar groups, whereas tryptamines are not observed in most virtual docking simulations to place their polar groups near TM3. These additional ligand classes were assayed for comparison. As mentioned for the previous specific aim, there is little empirical evidence upon which to base binding orientations of phenylalkylamines in the putative binding pocket of 5-HT_{2A} receptors. Other than the well supported interaction between the ligands' protonated amine and Asp3.32(155), the lack of experimental data is particularly true for interactions with TM3 residues. As the introduction, previous specific aims, and docking figures have indicated, the 2-methoxy of phenylalkylamines was predicted to interact with Ser3.36(159) and/or Thr3.37(160). Although S3.36(159) previously has been mutated in the rat 5-HT_{2A} receptor, those researchers focused mainly on the binding of tryptamines (Almaula *et al.*, 1996). No evidence could be found that T3.37(160) had been mutated in any serotonin receptor. Site directed mutagenesis of these two residues was utilized to investigate the hypotheses that either of these residues interact with the 2-methoxy group of phenylalkylamines.

4.5.1. Results

Human S3.36(159)A and T3.37(160)A mutant 5-HT_{2A} receptors possess acceptable affinity and expression levels

Saturation isotherm binding assays with wild type h5-HT_{2A} receptors utilizing [^{3}H]ketanserin and [^{125}I]DOI yielded K_D values of 1.1 ± 0.12 nM and 0.78 ± 0.01 nM, respectively, whereas these radioligands had K_D values of 2.23 ± 0.31 nM and 0.75 ± 0.12 nM, respectively, at T3.37(160)A mutant h5-HT_{2A} receptors. Due to the extreme shift in affinity, it was not feasible to determine a K_D value for [^{125}I]DOI at S3.36(159)A

mutant h5-HT_{2A} receptors, although [^{3}H]ketanserin yielded a K_D of 1.37 ± 0.18 nM. Receptor expressions based on B_{max} values from [^{3}H]ketanserin saturation binding assays were 8843 ± 858 fmol/mg for wild type h5-HT_{2A}, 2379 ± 79 fmol/mg for h5-HT_{2A}/S159A, and 2839 ± 486 fmol/mg for h5-HT_{2A}/T160A receptors.

Virtual docking of phenylalkylamines to an *in silico*-activated h5-HT_{2A} receptor homology model orients the ligands to indicate an interaction of the 2-methoxy with S3.36(159) and T3.37(160)

An examination of Figure 4.14 of Specific Aim 4 illustrates additional possible interactions of phenylalkylamines with receptor residues in the putative binding site of the h5-HT_{2A} receptor. In addition to interactions with the TM5 receptor residues explored in the previous specific aim, a number of interactions are observed with residues in TM3. Virtual docking simulations consistently oriented the protonated amine of these ligands near Asp3.32(155), the most highly conserved residue within this GPCR family, and shown to be important for the binding of both aminergic agonists and antagonists to 5-HT_2 receptors (Wang *et al.*, 1993; Kristiansen *et al.*, 2000). Most relevant to this specific aim, we further observed in the docking orientations of phenylalkylamines that the 2-methoxy was positioned near Ser3.36(159) and Thr3.37(160). Subsequent constrained dynamics and energy minimization simulations yielded reasonable distances and orientations to indicate a possible hydrogen bond, without affecting backbone structure or other well supported interactions, namely S5.43(239) to the 5-methoxy and F6.52(340) to the aromatic ring. Most of the highest ranked docking orientations of tryptamines did not indicate any direct interaction with either Ser3.36(159) or Thr3.37(160), as observed in Figure 4.10 of Specific Aim 3, although some lower ranked poses sometimes oriented the 4- or 5-oxygen of these ligands near these two residues.

The S3.36(159)A mutation in the h5-HT_{2A} receptor affects the binding and activity of nearly all ligands tested to a marked degree

As Table 4.14 indicates, the S3.36(159)A mutation in the h5-HT_{2A} receptor had dramatic effects on the binding affinity of nearly all compounds tested. LSD was the

least affected, but still had a 7-fold loss in affinity. Unexpectedly, serotonin was the most affected, with a greater than 40,000-fold loss in affinity. The other tryptamines, as well as the phenylalkylamines, had variable 40- to 2000-fold losses in affinity. The two N-Benzyls tested, 25I-NBOMe and 24-NBOMe, had about 1000- and 3000-fold losses in affinity, respectively. These changes in affinity represent losses of standard Gibbs free energy of binding ($\Delta\Delta G°$) much greater than that of the range of a hydrogen bond of -0.5 to -1.5 kcal/mol (Fersht, 1988) and are illustrated in Figure 4.18. The 2-ethyl analogue of DOM, 2-Et-DOM, did have less of a loss of binding energy than the 5-ethyl analogue 5-Et-DOM ($-\Delta\Delta G°$ -3.1 vs. -3.7).

Similar trends were observed with changes in functional potency due to the S3.36(159)A mutation, as indicated by Table 4.15, although in this assay system DOM was the most affected with about a 2000-fold loss in potency at PI hydrolysis. Serotonin had about a 200-fold loss, whereas the other two tryptamines tested, 5-MeO-DMT and psilocin, had 20- and 26-fold losses, respectively. We generally observed greater losses in potency for the phenylalkylamines than the tryptamines, however, the potency of 2-Et-DOM was detrimentally affected by this mutation more than 5-Et-DOM (450- vs. 200-fold). These relationships are illustrated in Figure 4.19.

2-Et-DOM and 5-Et-DOM were among the only compounds with intrinsic activities for PI hydrolysis detrimentally affected, although in this case 5-Et-DOM was affected more than 2-Et-DOM (ΔInt.Act. -65% vs. -36%). Mescaline and its analogues were the only other compounds tested that had changes to their intrinsic activities that were statistically discernable from wild type. Mescaline and escaline had shifts of about -60% whereas isoproscaline had the most dramatic decrease of about -80%.

The T3.37(160)A mutation in the h5-HT_{2A} receptor affects the binding and activity of tryptamines more than phenylalkylamines

As Table 4.14 indicates, the tryptamines were the only compounds that had lower affinity at the T3.37(160)A mutant h5-HT_{2A} receptor of about 10-fold. This loss corresponds to a decrease in standard Gibbs free energy of binding of -1 to -1.5 kcal/mol, within the range of the energetics of a hydrogen bond (Fersht, 1988). 2-Et-DOM and

mescaline and its analogues had 3-fold increases in affinity, whereas 5-Et-DOM and the other phenylalkylamines were not affected to a degree that was discernable from wild type. LSD was similarly unaffected. These relationships are illustrated in Figure 4.18.

A similar trend was observed for losses in functional potency for PI hydrolysis, as indicated by Table 4.15. Again, the tryptamines were most dramatically affected, with losses of about 15- to 65-fold. In this case, however, LSD was also affected and was 8-fold less potent in the functional assay. Most of the phenylalkylamine and related compounds were unaffected, with the exception of DOM, 25I-NBOMe, and 24-NBOMe, which had losses in functional potency of 3- to 5-fold. 25I-NBOMe and 24-NBOMe were also the only phenylalkylamine-related agonists with detrimental changes to intrinsic activity of 38% and 35%, respectively. These relationships are illustrated in Figure 4.19. No other phenylalkylamine had a change in intrinsic activities that was statistically discernable from wild type. Psilocin was the only tryptamine that had a loss of intrinsic activity (ΔInt.Act. -57%). Strangely, although the potency of LSD was unaffected, there was a loss of intrinsic activity (ΔInt.Act. -50%).

Table 4.14 Effects of the S3.36(159)A and T3.37(160)A mutations on binding to h5-HT_{2A} receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments. Except where indicated ([†]),mutant values were statistically distinguishable from wild-type, with $p<0.05$ for values of ΔpK_i obtained by ANOVA with Bonferroni post-tests comparing mutant values to wild type.

Drugs	(±)-[^{125}I]DOI		
	WT K_i (nM)	**S159A K_i (nM)**	**T160A K_i (nM)**
LSD	0.40 (0.02)	3.03 (0.20)	0.71 (0.14)[†]
5-HT	4.84 (0.2)	202100 (16900)	29.7 (1.0)
5-MeO-DMT	7.54 (1.06)	3650 (410)	95.3 (6.1)
psilocin	11.8 (1.2)	857 (57)	119 (15)
DOI	0.64 (0.06)	1300 (131)	0.66 (0.10)[†]
2CI	0.73 (0.06)	356 (22)	0.84 (0.12)[†]
DOM	5.91 (0.97)	212 (7)	7.01 (1.09)[†]
5-Et-DOM	22.4 (1.2)	12270 (400)	18.7 (1.2)[†]
2-Et-DOM	91.1 (9.7)	16140 (1040)	27.1 (2.6)
25I-NBOMe	0.04 (0.01)	48 (6)	0.027 (0.004)[†]
24-NBOMe	1.71 (0.34)	4600 (150)	1.75 (0.20)[†]
isoproscaline	470 (66)	139800 (56600)	174 (23)
escaline	610 (72)	22680 (6540)	222 (29)
mescaline	1500 (245)	349800 (126800)	471 (55)

[†] $p > 0.05$

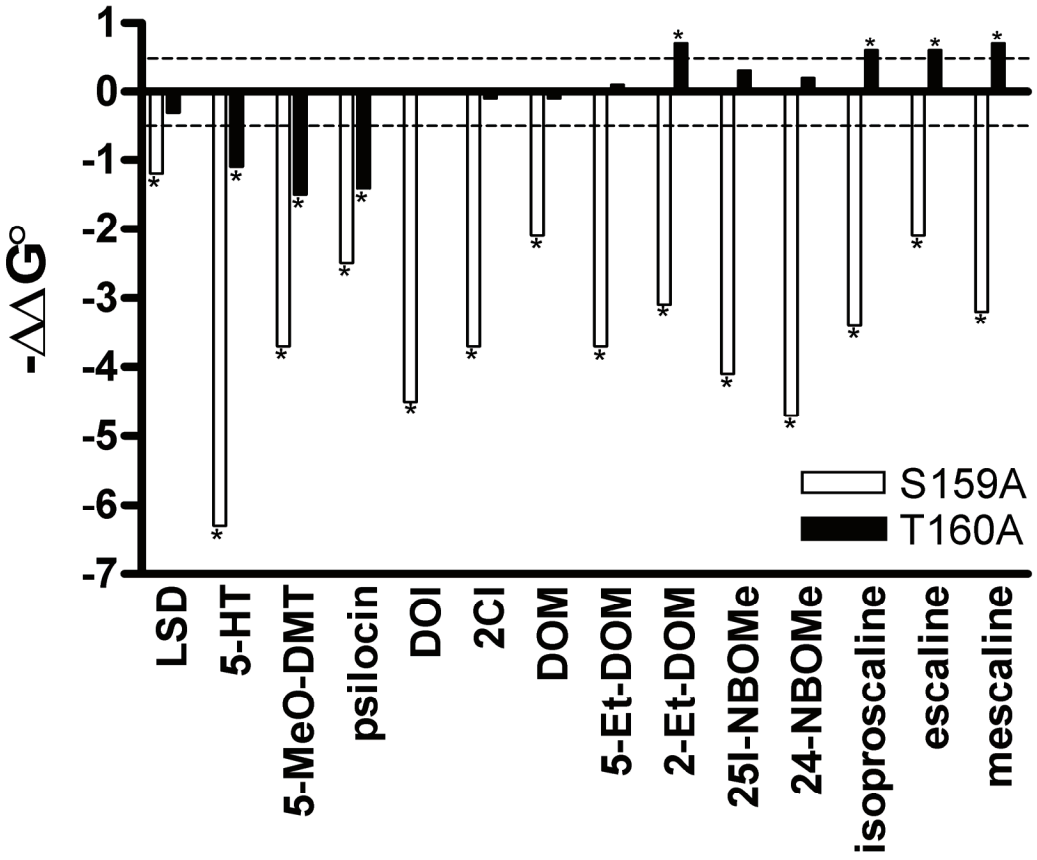


Figure 4.18 Effect of mutation of TM3 residues on the binding of agonists to h5-HT_{2A} receptors. Bars indicate changes in the standard Gibbs free energy of binding (ΔG°) for S3.36(159)A (open bars) and T3.37(160)A (closed bars) from the data of Table 4.14. Dashed lines at ±0.5 kcal/mol indicate the lower threshold for the energetics of a hydrogen bond (Fersht, 1988). * indicates $p < 0.05$ generated from two-way ANOVA with Bonferroni post-tests.

Table 4.15 Effects of the S3.36(159)A and T3.37(160)A mutations on h5-HT_{2A} receptor-mediated PI hydrolysis. Data are presented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity from at least three independent experiments. Except where indicated ([†]), EC50 values were statistically distinguishable from wild type as defined by $p<0.05$ from ANOVA calculations with Bonferroni post-tests between mutant and wild type receptors; * indicates $p<0.05$ for ΔInt.Act. values using the same statistical comparison.

Drug	WT h5-HT_{2A}		S159A		T160A	
	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)
LSD	0.22 (0.04)	84 (3)	0.47 (0.09)	102 (5)	1.8 (0.2)	34 (6)*
5-MeO-DMT	4.33 (0.78)	98 (4)	110 (7)	95 (3)	280 (28)	65 (12)*
5-HT	5.17 (0.97)	100 (3)	960 (84)	104 (1)	72 (9)	96 (1)
Psilocin	7.29 (0.72)	105 (9)	140 (8)	93 (1)	360 (69)	47 (10)*
DOM	2.81 (0.22)	87 (3)	6360 (830)	86 (3)	15 (2)	102 (8)
DOI	3.79 (0.36)	84 (2)	780 (25)	75 (2)	4.9 (0.4)[†]	86 (4)
2-Et-DOM	49.8 (6.96)	81 (2)	22230 (3790)	44 (3)*	103 (16)[†]	90 (9)
5-Et-DOM	71.9 (10.8)	84 (10)	15070 (2780)	18 (2)*	74 (12)[†]	88 (8)
25I-NBOMe	0.44 (0.07)	81 (4)	30 (5)	88 (1)	1.5 (0.3)	43 (14)*
24-NBOMe	4.00 (0.80)	89 (6)	710 (132)	75 (4)	17 (3)	54 (9)*
isoproscaline	220 (34)	101 (3)	33970 (6430)	19 (2)*	130 (5)[†]	95 (3)
escaline	275 (54)	100 (3)	142500 (8740)	42 (2)*	190 (17)[†]	95 (2)
mescaline	1120 (220)	83 (5)	167570 (14650)	28 (4)*	1140 (170)[†]	105 (8)

[†] EC50 $p > 0.05$; * Int.Act. $p < 0.05$.

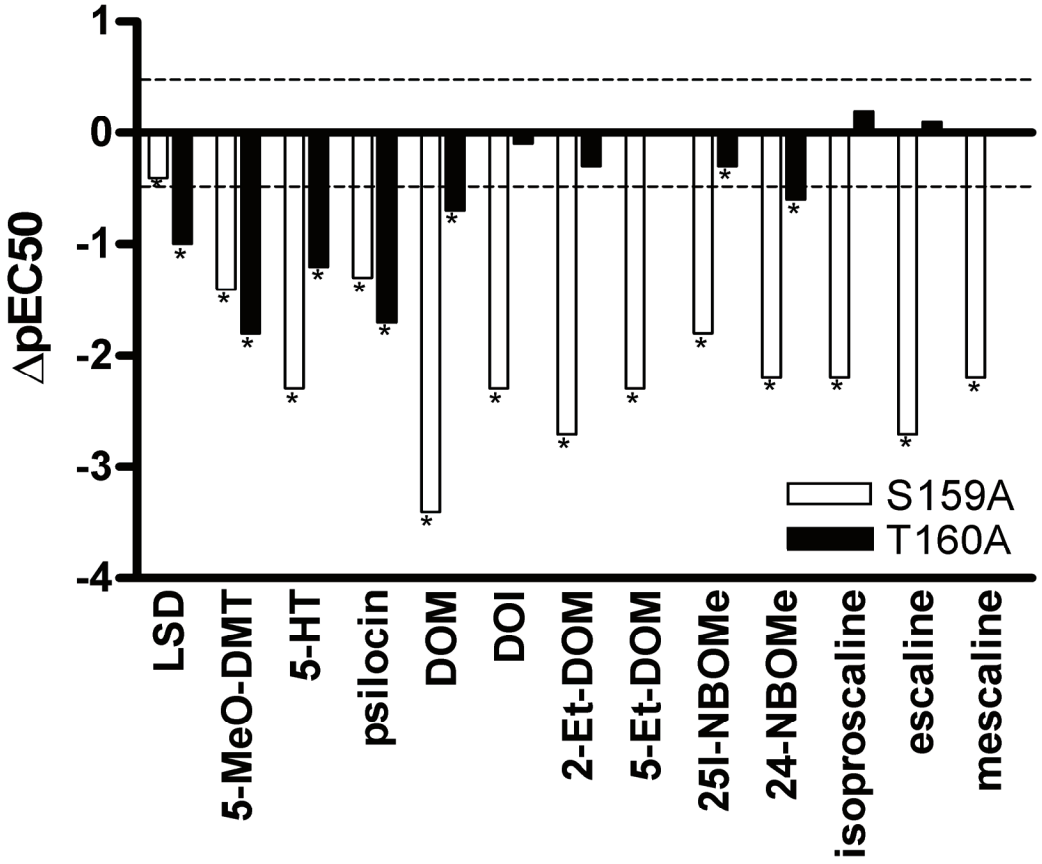


Figure 4.19 Effect of mutation of TM3 residues on the functional potency of agonists at h5-HT_{2A} receptors. Bars indicate log-scale normalized changes in the functional potency (pEC50) for S3.36(159)A (open bars) and T3.37(160)A (closed bars) from the data of Table 4.15. Dashed lines at ±0.5 kcal/mol indicate an arbitrary threshold for "weak" effects. * indicates $p<0.05$ generated from two-way ANOVA with Bonferroni post-tests.

4.5.2 Discussion

After gaining strong evidence for the receptor-ligand interactions, or lack thereof, indicated for several ligand classes with residues in TM5 and TM6 of the h5-HT_{2A} receptor, we wished to explore further the proposed binding interactions of these compounds by investigating interactions with TM3 receptor residues. We established in Specific Aim 3 and 4 that the 5-oxygen of phenylalkylamines and tryptamines is likely involved in a key interaction with Ser5.43(239), as indicated in our virtual docking orientations. Again, the optimal substitution for phenylalkylamines also includes a 2-methoxy group. As illustrated above, orientations from all virtual docking simulations indicate that all ligands are oriented so that the protonated amine can interact with Asp3.32(155), probably in a ion-ion salt bridge. These orientations have been observed previously (Chambers and Nichols, 2002), and are consistent with previous work showing that this highly conserved acidic residue is essential for the binding of aminergic ligands to this family of GPCRs (Strader *et al.*, 1988; Fraser *et al.*, 1989; Mansour *et al.*, 1992; Wang *et al.*, 1993; Javitch *et al.*, 1995; Kristiansen *et al.*, 2000).

As there was strong evidence of this ion-ion interaction, we were thus more interested in the hypotheses, displayed in Figures 4.2, 4.4 and 4.14, that docking orientations for phenylalkylamines and their analogues place the 2-methoxy in a position that may allow a hydrogen bond with Ser3.36(159) and/or Thr3.37(160), whereas highest ranked/lowest energy docking ensembles for tryptamines do not indicate any direct interaction with either of these residues. Previous mutagenesis of S3.36(159) in the rat 5-HT_{2A} receptor utilized only LSD and a few tryptamines and suggested that this residue was involved in hydrogen bonding to/sequestering of the protonated amine of the ligand (Almaula *et al.*, 1996). T3.37(160) has not been mutated in any serotonin receptor. Mutation of the cognate threonine residue to a cysteine in the dopamine D_2 receptor, T3.37(119), indicated that this residue may not be solvent accessible, even though antagonist binding affinity was decreased (Javitch *et al.*, 1995).

Similar to the previous two specific aims, non-disruptive "deletion" mutations of S3.36(159) and Thr3.37(160) to alanines in the h5-HT_{2A} receptor were each performed in

anticipation that they would eliminate specific receptor-ligand interactions without dramatically affecting global receptor structure (Fersht *et al.* 1987).

The dramatic effects of the S3.36(159)A mutation on affinity and activity of nearly all compounds, with no evident trend within ligand classes, as illustrated in Figures 4.18 and 4.19, would seem to indicate a possible global effect on protein structure. LSD is not affected to as marked a degree as the other compounds, however, and the K_D values for [^{3}H]ketanserin at wild type and h5-HT_{2A}/S159A mutant receptors are statistically indistinguishable (1.1 vs. 1.4 nM). Furthermore, even though all EC50 values are shifted, all compounds were able to stimulate the hydrolysis of radiolabeled phosphatidylinositides (PI). This finding would appear to suggest that, although an altered packing or orientation of helices may have occurred, affecting normal receptor activity, the receptor protein has folded to a state similar enough to wild type to be functional. Compounds tested at the h5-HT_{2A}/T160A receptor also were able to participate in radiolabeled displacement binding and PI hydrolysis in a dose-dependent manner. In contrast to the S3.36(159)A mutation, the T3.37(160)A mutation affects the various ligand classes differently. It is therefore likely that this mutant receptor has folded properly, and any difference in binding or function at this mutant receptor is likely due to the loss of a specific receptor-ligand interaction.

Unfortunately, neither of these mutations provided binding or functional assay results consistent with the hypothetical interaction of either residue with the 2-methoxy of phenylalkylamines. The S3.36(159)A mutation had dramatic effects on all ligands except for LSD. The extreme changes in the energetics of binding, as indicated in Figure 4.18, represent values much greater than those expected for a simple hydrogen bond of -0.5 to -1.5 kcal/mol (Fersht, 1988) and are not consistent with the previous idea that S3.36(159) may be only hydrogen bonding with the protonated amine (Almaula *et al.*, 1996). Moreover, consideration of these results fails to provide support for a direct interaction of S3.36(159) with the 2-methoxy of phenylalkylamines, contrary to our prediction. 2-Et-DOM appears to bind better than 5-Et-DOM, and both are more strongly affected by the S3.36(159)A mutation than DOM (Figure 4.18), but this relationship is reversed when functional potency is examined (Figure 4.19). Those findings may indicate a more

fundamental role for this residue, such as an inter- or intrahelical hydrogen bond, or interaction with a structural water that helps to stabilize an active state of the receptor. Indeed, even though the cognate residue in bovine rhodopsin is glycine G3.36(121), several structural waters are observed in the X-ray crystal structure in proximity to this residue and in between TM3 and TM4/TM6 (Okada *et al.*, 2004). Structural waters in bovine rhodopsin are believed to be important for the activity in all rhodopsin-like GPCRs (Okada *et al.*, 2002). Furthermore, this region of TM3 is believed to be essential for mediating the isomerization "switch" to active receptors states (Parnot *et al.*, 2000), although mutation of the cognate residues in α_{1B} and β_2 adrenergic receptors induced some pathway-selective constitutive activity (Perez *et al.*, 1996; Zuscik *et al.*, 1998) and allowed induction of transducin by rhodopsin in the dark (Han *et al.*, 1997).

Similarly inconsistent with the original hypotheses, the binding affinities of DOM and 5-Et-DOM are not affected by the T3.37(160)A mutation, although the affinity of 2-Et-DOM was slightly increased. DOM and the two N-Benzyls tested did have slight decreases in functional potency, but the rest of the phenylalkylamines were generally unaffected, whereas mescaline and its analogues had slightly increased potency. Furthermore, the tryptamines, which were predicted not to interact with this residue, had the most dramatic decreases in binding affinity and functional potency. This finding may indicate that alternate binding orientations or degenerate binding states may exist for these compounds. Low ranked docking orientations have been observed that place the polar ring substitution of tryptamines in proximity to S3.36(159) and/or T3.37(160) (data not shown) and may be consistent with some of the data indicating that the indoles of some tryptamines do not interact with S5.46(242) and that some tryptamines may adopt different binding orientations, as discussed in Specific Aim 4 and by others (Johnson *et al.*, 1997). Further experiments comparing polar and non-polar ring-substituted tryptamines at this mutant receptor may be necessary.

Similar to concerns about lack of information for structural waters with S3.36(159) discussed above, there is a lack of information about metal ion binding in our h5-HT_{2A} receptor homology model. Several zinc ions have been found in the crystal structure of bRho (Palczewski *et al.*, 2000; Okada *et al.*, 2002). Zinc ion binding near

E3.37(122) in bRho has been implicated as important for protein folding, retinal binding, and proper function of the receptor (Stojanovic *et al.*, 2004). It is unknown whether zinc coordination occurs in the transmembrane region at similar positions in other GPCRs.

We believe that these data, contrary to our original hypotheses, fail to support a direct interaction of either S3.36(159) or T3.37(160) with the 2-methoxy of phenylalkylamines. This conclusion does not necessarily invalidate the topology of the h5-HT_{2A} receptor observed in our *in silico*-activated homology model. Instead, these data help to remind us of the limitations of the model, and GPCR homology modeling in general. The lack of membrane solvation, metal ions, and structural waters in our model are obstructions to the more accurate calculations of receptor structure, movement, and ligand interaction. Hopefully, improvements of these factors in the future may help to identify better the role of S3.36(159) and T3.37(160) in receptor structure and receptor-ligand interactions. Further pharmacological experiments also are necessary, such as testing additional tryptamines at the T3.37(160)A mutant receptor or possibly determining whether reactive thiol groups in the 2-position of phenylalkylamines would react with a T3.37(160)C mutant receptor.

CHAPTER 5. CONCLUSIONS

The overarching goal of the hypotheses presented here was directed at the utilization, optimization, and validation of the *in silico*-activated homology model of the h5-HT_{2A} receptor developed previously in our laboratory (Chambers and Nichols, 2002). This model has proved useful in directing ligand synthesis to create high affinity agonists for the 5-HT_{2A} receptor (Chambers *et al.*, 2003; McLean *et al.*, 2006a; McLean *et al.*, 2006b) Moreover, as Specific Aim 1 shows, this model is very useful for qualitative explanations of differential pharmacological activity of structurally related compounds. The results, however, also illustrate a number of the limitations of these studies. In accounting for the effects of these compounds on humans, pharmacokinetic and/or drug metabolism factors are not taken into consideration. This restriction is a necessary but inherent flaw in a bottom-up approach of neuropharmacology, namely that a biophysical understanding of receptor activation and a molecular understanding of subsequent signaling processes are limited in their scope, scale, and systemic integration. Thus, as my colleague Jason Parrish more effectively explains (Parrish, 2006), one must be very careful in correlating activity at inositol phosphate release with overall subjective effects and activity of these compounds in humans. More relevant to the scope of this work, these results and methods illustrate the limitations of modeling techniques in general, namely artificially constraining atom-atom distances in virtual docking and subsequent energy simulations based on anticipated receptor-ligand interactions. In the early analysis of the work described in this thesis, some of the constraints utilized from previously indicated receptor-ligand interactions (Chambers and Nichols, 2002) appeared to have limited empirical support.

Artificial constraints are unfortunately a necessary part of current molecular modeling techniques (Pogozheva *et al.*, 2005). The primary factors affecting the

limitations of our homology model and the necessity of artificial constraints are: (1) the lack of accurate structural information for a GPCR with greater sequence identity to 5-HT receptors, particularly in the active state; (2) the inability to calculate hydrogen bond energies accurately; (3) the lack of protein (membrane) solvation in most energy simulations, particularly that of internal structural waters; and (4) the lack of accurate structural information for metal ion binding sites in 5-HT receptors. An NMR or X-ray crystal structure of a more highly related GPCR will greatly improve the accuracy of homology models of 5-HT receptors. Moreover, as docking algorithms and molecular field equations become more robust and include more computationally expensive energy calculations on faster computer platforms, the need for constraints on ligand position and orientation will hopefully diminish. Until that time, however, constraints are often necessary to maintain ligand-receptor interactions with strong empirical support and prevent dramatic changes in protein structure and ligand orientation or location.

The utilization, optimization, and validation of our h5-HT_{2A} receptor homology model thus becomes reliant on empirical support for the ligand-receptor interactions observed in our virtual docking simulations in order to justify the artificial constraints used in subsequent energy simulations. This goal actually highlights the overall utility of the model itself, that is as a hypothesis/lead generator for further research. In this manner, the pursuit of validation of the topology of our h5-HT_{2A} receptor model serves a dual purpose: justifying the utility of the model itself while furthering our understanding of the biophysical aspects of ligand-receptor interactions that lead to potent and selective compounds.

In Specific Aims 2 through 5, this thesis work employed a systematic approach to provide empirical support for the ligand-receptor interactions observed in the virtual docking orientations exemplified by Figures 4.4, 4.10, and 4.14. This work included the traditional 5-HT_{2A} receptor agonist ligand classes, namely tryptamines, ergolines, and phenylalkylamines, as well as a novel class of super potent agonists, the N-Benzyls. Unique structural differences between these classes, as well as slight changes of group substitution within classes, were utilized to explore the effects of specific receptor residue mutations. Furthermore, divergent responses to different ligand classes or group

substitution at a mutant receptor helped to provide support for the assumption that global receptor structure was not dramatically affected by these mutations. Finally, free energy relationships based on inferred equilibrium values of binding (K_i) and log scale normalized differences in functional potency (EC50) at wild type and mutant receptors were particularly useful in the analysis of the effects of these mutations on ligand-receptor interactions.

The data from these studies appear to suggest that a coordinated interaction between ligands and residues in TM3, TM5 and TM6 is necessary for agonist activity at the human 5-HT_{2A} receptor. In some cases, namely with the aromatic residues of TM6, Phe6.51(339) and Phe6.52(340), it is not appropriate to attribute specific roles for these residues in the activity and/or binding exclusively of agonists or antagonists, although both of these do appear critical for the binding and activity of the N-Benzyls. Other interactions are strongly supported and are implicated as being specifically important for agonist binding and activity, namely the interaction of Ser5.43(239) with the 4- or 5-oxygen of tryptamines and phenylalkylamines. The interaction of Ser5.46(242) with the indole nitrogen of tryptamines and lack of interaction with phenylalkylamines, as well as a lack of interaction of Ser5.43(239) with ergolines, is similarly supported, although not as strongly. There are indications that tryptamines may be able to adopt alternate binding orientations and still maintain active receptor states, particularly tryptamines without polar ring substituents. As the interaction with S5.43(239) appears much stronger and more important to binding and activity than with S5.46(242), it may be possible that an interaction between S5.46(242) and the indole nitrogen of the tryptamines is only favorable when S5.43(239) is first engaged. The mutation of Gly5.42(238) to an alanine seems to support further the binding orientations observed in virtual docking simulations of phenylalkylamines, particularly mescaline and its analogues.

Not all the data from these studies, however, support the orientations and interactions observed in virtual docking simulations to the h5-HT_{2A} receptor homology model. The mutation of Asn6.55(343) does not affect the binding or activity of ergolines (Chambers and Nichols, 2002) and N-Benzyls as expected. Rather, a number of smaller classic ligands were most affected, as well as N-Benzyl compounds without polar benzyl

ortho-substitution. These findings may, however, support the hypothesis that tryptamines can adopt alternate binding orientations, as the polar ring substituted tryptamines were among those most greatly affected by this mutation. It is also possible that residues in the loop connecting TM4 and TM5 (EL2), may be contacting the ergolines and N-Benzyls, as this loop has been shown to interact with agonists and antagonists in the ligand binding site of other related GPCRs (Olah *et al.*, 1994; Audoly and Breyer, 1997; Ott *et al.*, 2002; Seong *et al.*, 2003; Shi and Javitch, 2004; Kukkonen *et al.*, 2004). Accurate modeling of this loop is difficult, as much of it is exposed to the aqueous extracellular space, is generally of a random secondary structure, and accurate solvation is problematic. The high variability and low sequence identity make it nearly impossible to generate accurate homology models of the loop regions of GPCRs. Additional refinements of the EL2 loop structure and experiments to test both of the hypotheses of interaction of N-Benzyls and ergolines with this loop are necessary (see Future Directions).

Support for the interactions of the 2-methoxy of phenylalkylamines with polar residues on TM3, namely Ser3.36(159) and Thr3.37(160), are similarly inconclusive. The tryptamines are surprisingly affected by mutations of these residues, again possibly indicating alternate binding orientations for these compounds. The effects on binding and activity of all compounds resulting from the S3.36(159)A mutation seem to indicate that this residue plays a more fundamental role than simply hydrogen bonding to the ligands. As discussed in Specific Aim 5, S3.36(159) may be important for coordinating structural waters, and T3.37(160) may be involved in zinc ion binding in this region, a feature that is difficult to model accurately.

In summary, the majority of the data from this work provides relatively strong evidence in support of the orientations observed in virtual docking simulations of classic and novel ligands to our h5-HT_{2A} receptor homology model. A cartoon of these interactions is provided in Figure 5.1. The use of artificial constraints in energy simulations reflective of the interactions with S5.43(239) and S5.46(242) are justified by these results, similar to results with D3.32(155) from previous work (Strader *et al.*, 1988; Fraser *et al.*, 1989; Mansour *et al.*, 1992; Wang *et al.*, 1993; Javitch *et al.*, 1995; Kristiansen *et al.*, 2000). Data from the S3.36(159)A and T3.37(160)A mutants,

however, were not consistent with the simple hydrogen bonding interactions suggested by the modeling. Nonetheless, previous studies have supported a limited role in utilizing the h5-HT_{2A} receptor homology model for directing ligand synthesis (Chambers, 2002; Chambers *et al.*, 2003; McLean *et al.*, 2006a; McLean *et al.*, 2006b), indicating a generally high accuracy of the model structure.

Overall, this work has expanded our understanding of the biophysical aspects of ligand-receptor binding to and activation of the human serotonin 2A receptor, in that coordinated and efficient contacts with TM3, TM5, and TM6 receptor residues are essential for high affinity and potent activity at these receptors. These findings can be extended to a number of related Family A monoamine-binding GPCRs, due to the homology and implied shared structure of these receptors. Finally, this work is consistent with the topology of our *in silico*-activated h5-HT_{2A} receptor homology model and highlights both its utility and limitations for providing insight and research leads into these biophysical phenomena. Additional GPCR experimental structures, along with further refinements to molecular modeling techniques, will lead to models with improved accuracy. Hopefully the current work can ultimately be integrated and expanded into pursuing such questions such as the biophysical basis for second messenger pathway selectivity of 5-HT_{2A} receptor agonists.

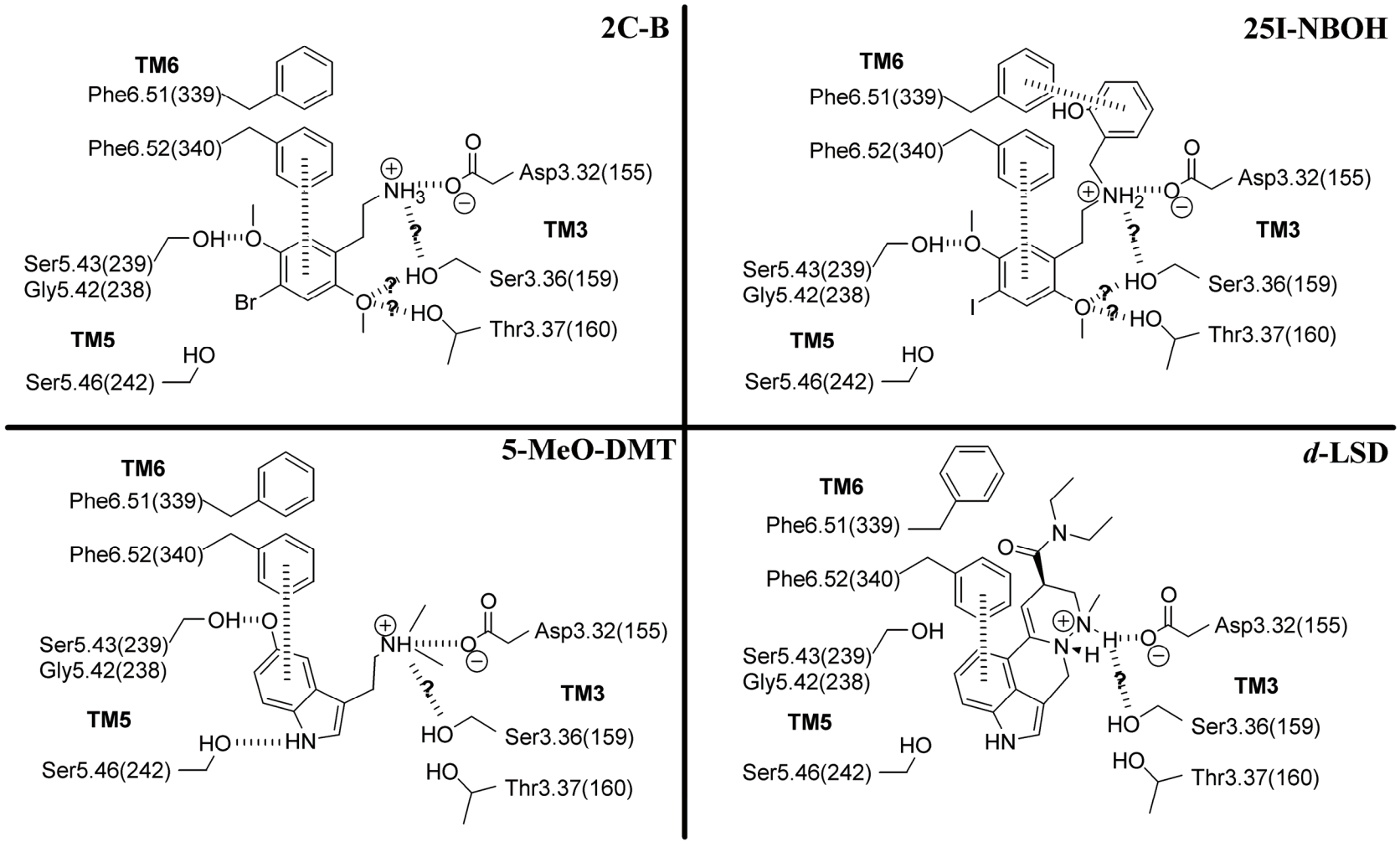


Figure 5.1 Cartoon of binding interactions observed in virtual docking simulations. Hydrogen bond and π–π interactions strongly supported by this or other works are indicated by a hashed line, whereas weakly or unsupported interactions are indicated by a hashed line overlaid with a question mark.

CHAPTER 6. FUTURE DIRECTIONS

As with any good scientific work, of the few questions that have been answered here, many more have been generated. First and foremost, all of the functional assays for these experiments relied primarily on measurement of radiolabeled inositol phosphates, presumably arising from G_q activation of PLC-mediated hydrolysis of phosphatidyl-inositol (PI). A more complete picture of how structurally distinct agonists differentially activate other signaling systems is necessary, especially the eicosanoid release mediated by human 5-HT_{2A} receptor activation (see Parrish, 2006). At the very least, more compounds need to be assayed for total eicosanoid release due to human 5-HT_{2A} receptor activation, although measurements of particular eicosanoids, namely 2-AG and arachadonic acid, would be preferable. Any of the mutations presented here could then be assayed for their effect on the selectivity of second messenger signal generation.

Additional compounds need to be tested at the mutant receptors created for this work. More tryptamines, particularly ring-unsubstituted and N(1)-alkyl substituted tryptamines, should be tested at h5-HT_{2A} receptors with the N6.55(343)A and T3.37(160)A mutations to test further whether some tryptamines may adopt alternate binding orientations that interact with these residues and stabilize degenerate active receptor states. Additional ergolines require testing at the S5.43(239)A and S5.46(242)A mutant receptors to verify the effects observed with LSD.

Additional mutations and changes to ligand structure also are needed. Increasing the steric bulk of the mutations of G5.42(238) and T3.37(160) by changing these residues to valine instead of alanine was attempted, but I was unable to establish cell lines with stable expression. Cell lines that survived antibiotic selection and appeared to bind [^{3}H]-ketanserin or [^{125}I]-DOI yielded affinities and expression levels that were too variable to establish reliable mean values (data not shown). These mutations/stable cell lines may

need to be repeated, as they may further help to confirm the localization of the 4-substituent of phenylalkylamines, in the case of G5.42(238)V, or the interaction with some tryptamines, in the case of T3.37(160)V.

A further understanding of the role of the loop connecting TM4 and TM5 of the h5-HT_{2A} receptor (EL2) in agonist binding and activity may also be interesting. One method would be to carry out scanning cysteine accessibility studies with residues in EL2. Each cysteine residue could then be examined for its ability to react with larger 5-HT_{2A} receptor agonists with reactive thiol groups on parts of the molecule closer to the extracellular side of their predicted binding orientations, namely on or near the carbonyl substitution of ergolines and on the *N*-arylmethyl moiety of the N-Benzyls. Additionally, this loop may be involved in the selectivity of some agonists for h5-HT_{2A} over h5-HT_{2C} receptors, namely the N-Benzyl compounds. A h5-$HT_{2A/2C}$ receptor chimera with the EL2 transposed also might be useful in the exploration of compounds previously shown to have h5-HT_{2A} receptor selectivity.

Finally, refinements to the modeling techniques utilizing the h5-HT_{2A} receptor homology model are necessary. I anticipate that the following features, although non-trivial, would greatly improve the accuracy of the model: (1) solution of an X-ray crystal structure of a GPCR with high(er) sequence identity to 5-HT receptors; (2) solvation of the transmembrane domains in a simulated lipid bilayer environment; (3) restoring the removed loops, solvation of internal and extracellular loops in an aqueous environment, and *de novo* modeling of their structure; (4) addition of structural waters to the model; (5) addition of metal ions to the ligand binding site; and (6) utilization of a molecular force field that accurately calculates hydrogen bond energies and/or utilizes more accurate classical parameterization of hydrogen bonds.

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APPENDIX

APPENDIX

Binding affinities for all compounds at all receptors

In the following pages of this appendix, I have attempted to provide a centralized source for the binding affinities of all the compounds I tested at every receptor where they were tested. It was not always feasible to include or discuss some of these values in the specific aims of the main text. Figure A.1 displays additional structures not illustrated in Figures 4.1, 4.3, 4.7, 4.9, and 4.13. Table A.1 displays binding affinities for compounds at wild type human and rat 5oh, -HT 1A, 2A, and 2C receptors. Table A.2 displays binding affinities at human 5-HT_{2A} receptors with mutations of TM3 or TM5 residues. Table A.3 displays binding affinities at human 5-HT_{2A} receptors with mutations of TM6 residues. As indicated by superscript letters, some data by other former members of our laboratory are included to aid comparison.

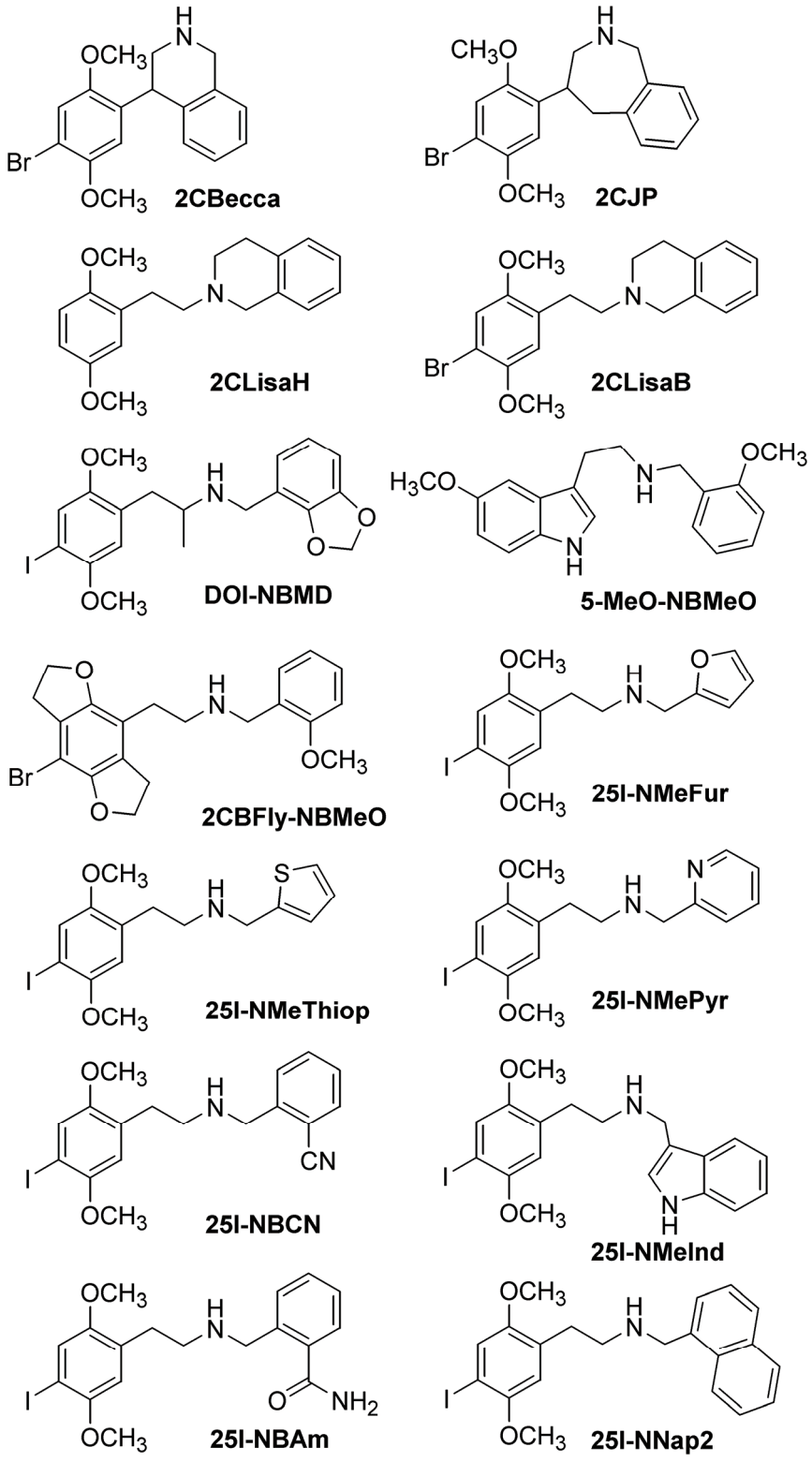

Figure A.1 Additional structures for binding assays.

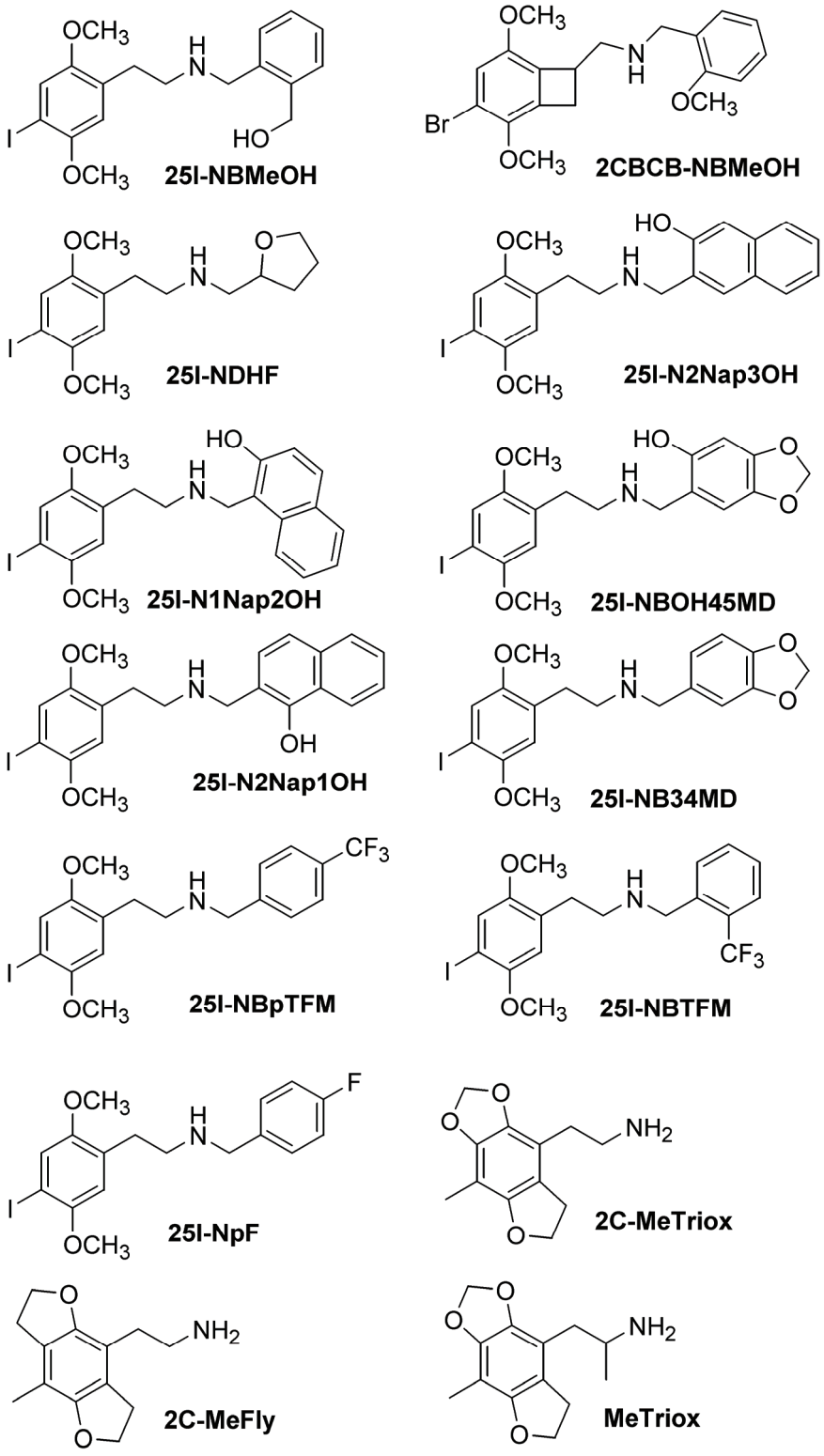

Figure A.1 (continued) Additional structures for binding assays.

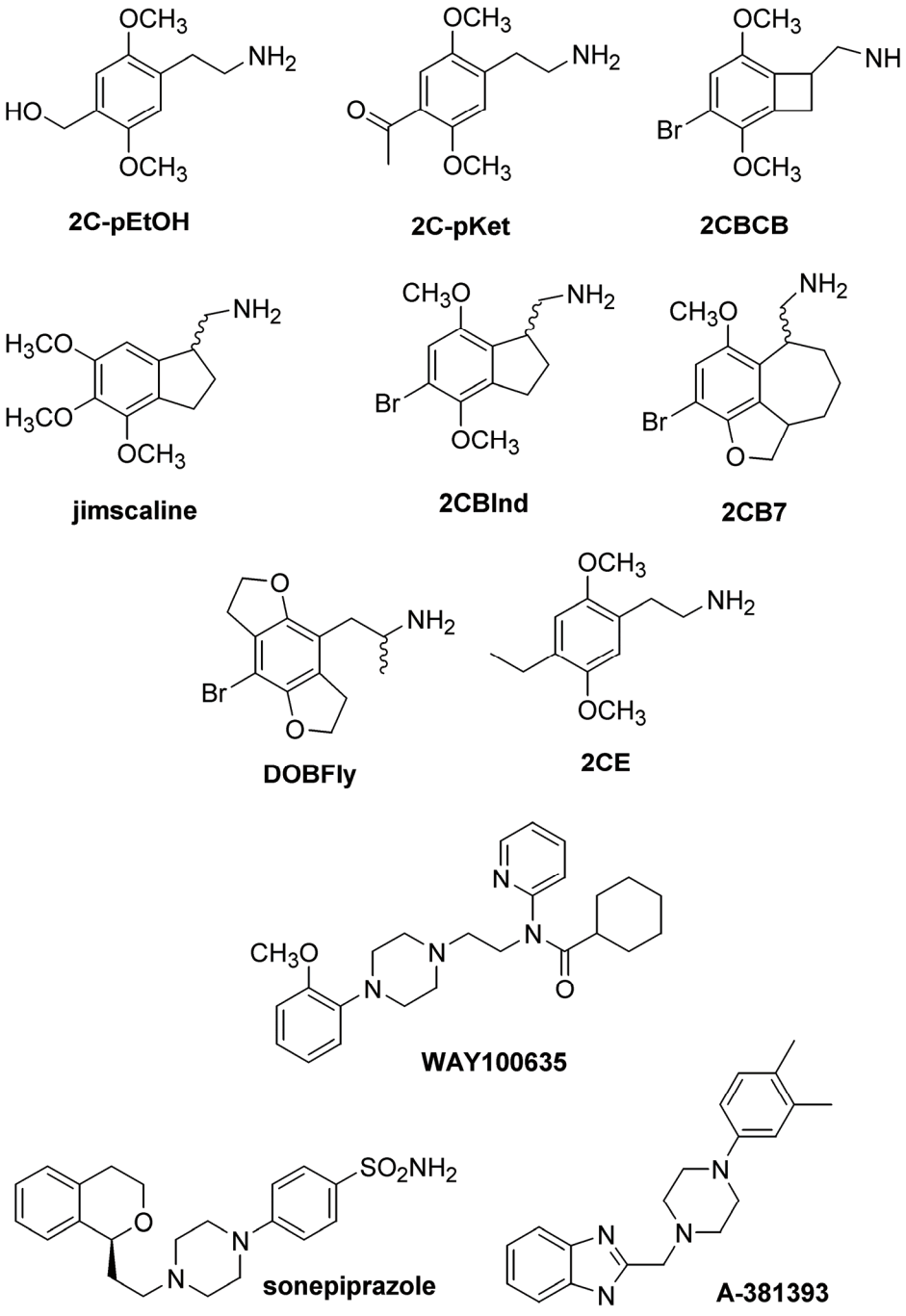

Figure A.1 (continued) Additional structures for binding assays.

Table A.1 Binding affinities at wild type human and rat 5-HT receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments. Superscripted notes indicate that values are from another source/person: a - (Monte *et al.*, 1997); b - (Blair *et al.*, 2000); c - (Nichols *et al.*, 2002); d – (Marona-Lewicka *et al.*, 2002); e - Parrish, JC; f – Kurrasch-Orbaugh, D; g- (Parrish *et al.*, 2005).

Table A.1

Drugs	[^{3}H]8-OH-DPAT	[^{125}I]DOI				[^{3}H]Ketanserin
	h5-HT$_{1A}$	r5-HT$_{2A}$	r5-HT$_{2C}$	h5-HT$_{2A}$	h5-HT$_{2C}$	h5-HT$_{2A}$
LSD	1.10 (0.01)[c]	3.5 (0.62)[c]	5.5 (0.31)[c]	0.40 (0.02)	3.62 (0.51)	0.81 (0.16)
LA-Pip	0.45 (0.09)[e]	2.6 (0.1)[e]	2.3 (0.2)[e]	0.32 (0.02)		
Lamide	2.1 (0.4)[e]	3.2 (0.1)[e]	7.4 (0.4)[e]	0.33 (0.066)		
S,S-trans	0.45 (0.01)	8.3 (1.7)c	6.5 (0.15)[c]	0.54 (0.11)		
5-HT	0.98 (0.07)[d]	20.8 (2.8)[f]	1.51 (0.31)[f]	4.84 (0.2)	37.7 (7.2)	77.6 (13.8)
psilocin	48.8 (5.5)[b]	25 (4.7)[b]	10 (1.4)[b]	11.8 (1.2)	29.6 (5.3)	22.8 (4.0)
5-MeO-DMT	1.74 (0.08)[b]	42.2 (9.9)[b]	16 (1.8)[b]	7.54 (1.06)	219 (17)	49.2 (3.2)
mescaline	4142 (296)	551 (35)[a]	303 (3)[a]	1499 (245)	2438 (177)	14640 (2447)
(±)-DOI	2604 (292)	0.65 (0.12)[g]	0.80 (0.08)	0.64 (0.06)	1.71 (0.30)	4.13 (0.11)
2CI (25I)	123 (24)	0.65 (0.07)[g]	1.22 (0.03)	0.73 (0.06)	1.82 (0.20)	4.52 (0.30)
2CH (25H)	114 (8)[e]	227 (39)[e]	55 (0.6)[e]	377 (67)		1999 (311)
25H-NMe	247 (23)[e]	1286 (64)[e]	206 (34)[e]	1907 (254)		5934 (92)
25H-NPr	879 (64)[e]	734 (30)[e]	656 (127)[e]	1295 (151)		3597 (642)
25H-NB	6973 (861)[e]	17.5 (1.9)[e]	14.7 (1.9)[e]	68.1 (10.6)		184 (33)
25I-NB	2205 (106)	0.31 (0.03)[e]	1.15 (0.90)[e]	0.25 (0.05)	1.08 (0.24)	0.28 (0.02)
25H-NBOMe	6973 (1001)[e]	1.19 (0.17)[e]	1.01 (0.07)[e]	2.83 (0.31)		11.0 (0.5)
25H-NBOH	4962 (146)[e]	2.76 (0.40)[e]	6.54 (0.64)[e]	3.73 (0.45)		11.6 (1.7)
25I-NBOMe	1696 (311)[e]	0.09 (0.010)[e]	0.13 (0.02)[e]	0.044 (0.006)	0.43 (0.08)	0.15 (0.03)
25I-NBOH	2749 (210)[e]	0.12 (0.02)[e]	0.21 (0.02)[e]	0.061 (0.012)	0.13 (0.01)	0.068 (0.012)
24DMP	920 (93)[e]	202 (19)[e]	113 (15)[e]	298 (29)		999 (182)
24-NB	13790 (585)[e]	28.5 (2.9)[e]	44 (1.7)[e]	26.6 (2.7)		71.9 (3.0)
24-NBOMe	12219 (2212)[e]	0.68 (0.12)[e]	1.23 (0.24)[e]	1.71 (0.34)	7.73 (1.54)	5.24 (1.01)
24-NBOH	9488 (670)[e]	0.67 (0.01)[e]	3.25 (0.66)[e]	1.51 (0.20)	17.2 (2.8)	2.83 (0.36)
25I-NBF	3803 (170)	0.28 (0.04)[e]	0.85 (0.11)[e]	0.26 (0.05)	2.36 (0.41)	0.19 (0.03)
2CI-N(2)Nap	1137 (152)	3.74 (0.52)[e]	176 (30)	4.83 (0.55)	38.9 (7.6)	6.68 (1.02)
25I-NBMD	971 (69)	0.193 (0.022)	0.41 (0.07)	0.049 (0.008)	1.70 (0.23)	0.21 (0.03)

Table A.1 (continued)

Drugs	[^{3}H]8OHDPAT	[^{125}I]DOI				[^{3}H]Ketanserin
	h5-HT$_{1A}$	r5-HT$_{2A}$	r5-HT$_{2C}$	h5-HT$_{2A}$	h5-HT$_{2C}$	h5-HT$_{2A}$
2CBecca		53.3 (9.3)	236 (15)	38.8 (3.3)	267 (50)	63.4 (5.7)
2CJP		19.4 (0.6)	227 (38)	36.5 (2.0)	318 (46)	25.9 (2.2)
2CLIsaH		690 (64)	1404 (145)	1158 (214)	1303 (150)	
2CLIsaB		45.0 (7.2)	270 (42)	50.2 (8.4)		
(±)-DOI-NBMD		0.27 (0.02)	0.43 (0.08)	0.29 (0.05)	3.14 (0.30)	
5-MeO-NBOMe		2.89 (0.57)	2.08 (0.32)	1.49 (0.31)	9.90 (1.30)	
2CBFly-NBOMe		0.14 (0.03)[e]	0.26 (0.05)[e]			
25I-NMeFur		0.78 (0.12)	0.99 (0.1)			
25I-NMeThiop		0.45 (0.09)	0.59 (0.06)	1.02 (0.09)		
25I-NMePyr		3.45 (0.7)	5.81 (1.14)			
25I-NBCN		276 (65)	23.2 (4.1)			
25I-NMeInd		2.67 (0.49)	11.8 (1.7)			
25I-NBAm		0.84 (0.1)	0.73 (0.09)	1.18 (0.22)		
25I-N1Nap		1.07 (0.11)	14.0 (1.5)			
25I-NBMeOH		0.44 (0.03)	0.43 (0.01)	0.79 (0.05)		
2CBCB-NBOMe		0.220 (0.004)	0.46 (0.03)	0.27 (0.01)		
25I-NBDHF				0.026 (0.006)	1.03 (0.15)	
(±)-DOI-NBOMe				0.78 (0.04)	21.0 (3.4)	
25I-N2Nap3OH				664 (18)		
25I-N1Nap2OH				3552 (3207)		
25I-NB34MD				0.69 (0.05)		
25I-NBOH45MD				0.82 (0.17)		
25I-N2Nap1OH				0.45 (0.06)		
25I-NBpTFM				205 (44)		
25I-NBTFM				1.31 (0.15)		
25I-NBpF				37.3 (6.0)		

Table A.1 (continued)

Drugs	[^{3}H]8OHDPAT	[^{125}I]DOI				[^{3}H]Ketanserin
	h5-HT$_{1A}$	r5-HT$_{2A}$	r5-HT$_{2C}$	h5-HT$_{2A}$	h5-HT$_{2C}$	h5-HT$_{2A}$
(*R*)-DOB		0.27 (0.05)[e]		0.29 (0.04)	0.62 (0.05)	
(*S*)-DOB		1.49 (0.27)[e]		1.86 (0.18)	2.87 (0.21)	
(±)-DOB		0.66 (0.11)[g]		0.68 (0.10)		
2CB		0.66 (0.13)[g]		0.89 (0.04)		
(*R*)-DOI		0.31 (0.03)[g]		0.27 (0.02)		
(*S*)-DOI		0.98 (0.17)[g]		0.90 (0.04)		
DOTFM		0.61 (0.09)[g]		0.46 (0.02)		
2CTFM		0.65 (0.10)[g]		0.69 (0.04)		
Aleph2		1.78 (0.30)[g]		0.70 (0.05)		
2CT2		1.81 (0.23)[g]		0.78 (0.02)		
(*R*)-TFMFly		0.15 (0.01)[g]		0.12 (0.01)		
(*S*)-TFMFly		0.34 (0.05)[g]		0.49 (0.03)		
2C-MeTriox		2.14 (0.21)		10.35 (1.1)	2.84 (0.47)	
2C-MeFly		1.29 (0.11)		6.22 (0.74)	1.91 (0.51)	
2C-pEtOH		344 (61)	338 (61)	427 (68)	513 (62)	
2C-pKet		91.1 (13.4)	93.1 (3.3)	83.8 (14.1)	150 (25)	
(±)-MeTriox		0.60 (0.08)	0.56 (0.07)	6.33 (0.61)	4.87 (0.25)	
(±)-2C-BCB		0.75 (0.09)[e]		0.73 (0.12)		
(*R*)-2CBCB		0.35 (0.01)[e]		0.26 (0.00)		
(*S*)-2CBCB		15 (2.7)[e]		42.0 (0.6)		
jimscaline		130 (20)[e]	60 (10)[e]			
(*R*)-jimscaline		69 (6)[e]			243 (27)	
(*S*)-jimscaline		1120 (180)[e]		3129 (492)		
(±)-2CBInd		53 (2.9)[e]		47.1 (6.6)		
(±)-syn-2CB7		170 (26)[e]		73.9 (4.7)		
(±)-anti-2CB7		200 (16)[e]		173 (11)		

Table A.1 (continued)

Drugs	[^{3}H]8OHDPAT	[^{125}I]DOI				[^{3}H]Ketanserin
	h5-HT$_{1A}$	r5-HT$_{2A}$	r5-HT$_{2C}$	h5-HT$_{2A}$	h5-HT$_{2C}$	h5-HT$_{2A}$
(*R*)-DOBfly				0.27 (0.02)	0.15 (0.03)	
(*S*)-DOBfly				1.13 (0.08)	0.58 (0.03)	
2CE				1.39 (0.12)	1.61 (0.17)	
escaline				611 (72)	4366 (3540)	
isoproscaline				465 (66)		
TMA2				133 (18)		
DET				64.9 (1.3)		
(±)-DOM				5.91 (0.97)		
5-Et-DOM				22.4 (1.2)		
2-Et-DOM				91.1 (9.7)		
5-H-DOM				169 (12)		
5-H-2CD				169 (13)		
(±)-DOH (25DMA)				245 (28)		
5-MeO-(N1)-iPr-T				494 (91)		
5-MeO-T				1.34 (0.22)		
tryptamine				29.7 (4.4)		
5-Me-T				11.7 (0.6)		
DMT				75.1 (6.0)		
N1-methylserotonin				70.0 (1.2)		
WAY100635				604 (103)		
sonepiprazole				5290 (461)		
A-381393				1230 (21)		

Notes: a - Monte *et al.* 1997; b - Blair *et al.* 2000; c - Nichols *et al.* 2002; d – Marona-Lewicka *et al.* 2002; e - Parrish, JC; f – Kurrasch-Orbaugh, D; g- Parrish *et al.* 2005.

Table A.2 Binding affinities at TM3 and TM5 mutant h5-HT_{2A} receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments.

Drugs	[^{125}I]DOI K_i (nM)				
	3.36 S159A	**3.37 T160A**	**5.42 G238A**	**5.43 S239A**	**5.46 S242A**
LSD	3.03 (0.20)	0.71 (0.71)	0.25 (0.05)	0.41 (0.08)	1.66 (0.32)
5-HT	> 10 μM	29.7 (1.0)	7.8 (1.3)	53.2 (8.0)	20.2 (0.9)
psilocin	857 (57)	119 (15)	19.1 (4.1)	58.0 (3.7)	23.1 (3.3)
5-MeO-DMT	3654 (414)	95.3 (6.1)	5.5 (0.7)	105 (19)	36.0 (1.9)
mescaline	> 10 μM	471 (55)	692 (54)	2023 (385)	456 (58)
escaline	> 10 μM	222 (29)	853 (31)	526 (57)	109 (33)
isoproscaline	> 10 μM	174 (23)	2287 (380)	1628 (203)	157 (26)
(±)-DOM	212 (7)	7.01 (1.09)	3.73 (0.59)	46.4 (5.9)	5.04 (0.55)
5-Et-DOM	> 10 μM	18.7 (1.2)	43.3 (0.8)	32.2 (1.9)	31.5 (5.0)
2-Et-DOM	> 10 μM	27.1 (2.6)	44.2 (8.4)	460 (92)	38.5 (4.1)
5-H-DOM			108 (14)	343 (41)	164 (6.53)
5-H-2CD				250 (30)	
(±)-DOH			30.0 (2.4)	2246 (144)	294 (54)
(±)-DOI	1300 (131)	0.66 (0.10)	0.64 (0.09)	2.19 (0.26)	0.83 (0.11)
2CI (25I)	356 (22)	0.84 (0.12)	0.74 (0.08)	1.59 (0.27)	1.28 (1.28)
2CH (25H)			43.7 (7.9)	782 (19)	440 (23)
24DMP				602 (85)	
24-NBOMe	4597 (149)	1.75 (0.20)	0.61 (0.16)	1.8 (0.27)	1.97 (0.38)
24-NBOH					
25I-NBOMe	48 (6)	0.027 (0.004)	0.07 (0.01)	0.11 (0.02)	0.12 (0.01)
5-MeO-iPr-T				786 (85)	24.1 (0.8)
5-MeO-T				4.17 (0.68)	2.70 (0.29)
tryptamine				50.2 (6.6)	36.0 (6.0)
5-Me-T				10.9 (1.3)	25.8 (3.9)
DMT				116 (12)	142 (16)
N1-Me-5-HT				320 (37)	5.73 (0.83)
TMA2					142 (29)
2CE					1.61 (0.22)

Table A.3 Binding affinities at TM6 mutant h5-HT_{2A} receptors. Data are presented as the mean and (SEM) in nM of K_i values from nonlinear regression fits of a single binding site model from at least three independent experiments.

Drugs	[^{125}I]DOI	[^{3}H]Ketanserin	[^{125}I]DOI
	6.51 F339L	**6.52 F340L**	**6.55 N343A**
LSD	0.60 (0.12)	13.0 (1.1)	0.20 (0.031)
LA-Pip			0.47 (0.09)
Lamid			0.36 (0.06)
S,S-trans			0.36 (0.04)
5-HT	59.6 (10.0)	> 10 μM	61.3 (7.0)
psilocin	28.6 (4.3)	3659 (243)	15.78 (1.51)
5-MeO-DMT	129 (15)	> 10 μM	6.91 (0.94)
DMT			54.0 (6.5)
DET			51.4 (8.7)
mescaline	4488 (608)	> 10 μM	7357 (514)
escaline			955 (182)
isoproscaline			1858 (331)
(±)-DOB			3.53 (0.50)
2CB			1.39 (0.14)
(±)-DOM			25.1 (3.6)
5-Et-DOM			13.8 (1.7)
2-Et-DOM			192 (34)
(±)-DOI	2.78 (0.39)	309 (29)	0.72 (0.08)
2CI (25I)	2.63 (0.32)	28.9 (4.8)	1.23 (0.19)
2CH (25H)	5786 (734)	> 10 μM	
24DMP (24)	1013 (190)	8391 (1200)	

Table A.3 (continued)

Drugs	[^{125}I]DOI	[^{3}H]Ketanserin	[^{125}I]DOI
	6.51 F339L	**6.52 F340L**	**6.55 N343A**
24-NB	1768 (339)	3316 (356)	
24-NBOMe	252 (49)	703 (14)	
24-NBOH	306 (57)	292 (14)	
25H-NMe	8719 (671)	> 10 μM	3.01 (0.57)
25H-NPr	7863 (769)	9815 (943)	3.68 (0.70)
25H-NB	2722 (470)	6698 (1031)	
25H-NBOMe	1435 (192)	689 (107)	
25H-NBOH	2642 (455)	277 (40)	
25I-NB	3.15 (0.74)	27.0 (1.8)	0.631 (0.12)
25I-NBOMe	2.08 (0.35)	4.30 (0.76)	0.067 (0.010)
25I-NBOH	1.84 (0.16)	1.58 (0.17)	0.049 (0.005)
25I-NMeNap	157 (31)	268 (268)	6.10 (0.63)
25I-NNap2OH			0.86 (0.09)
25I-NBMD	0.29 (0.03)	0.94 (0.17)	0.084 (0.017)
25I-NBF	15.2 (1.7)	37.9 (1.3)	0.64 (0.13)
2CBecca	219 (39)	33.6 (4.9)	
2CJP	106 (21)	993 (195)	

VITA

VITA

Michael Braden was born on February 28th 1977 to Charles Goetzman Braden, Jr. and Katherine Faucette Braden in Honolulu, Hawai'i. Growing up in Maunawili on the windward-side of the island of Oahu, hiking and playing in the ocean frequently, gave Michael an early appreciation of nature. Upon graduating from Punahou School as the Executive Officer of the school's Army JROTC battalion with an early, strong interest in science, particularly neuroscience and pharmacology, Michael pursued a Bachelor of Science degree in Biochemistry from the Chemistry Department of the University of Oregon in Eugene. As well as working part-time during his undergraduate studies, Michael did two years of post-graduate research at Marker Gene Technologies, a small biotech research company in Eugene, Oregon under the direction of Dr. John Naleway. Michael came to Purdue University specifically to work with Dr. David Nichols in the pursuit of exploring the mechanism of action of hallucinogens at the molecular level. In early 2007, Michael accepted a post-doctoral research fellowship with Dr. John Gerdes at the University of Montana in Missoula to do molecular modeling work.